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The Role of miRNAs in the Progression of Prostate Cancer from Androgen-Dependent to Androgen-Independent Stages

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14. ABSTRACT

The androgen receptor (AR) stimulates and represses gene expression to promote the initiation and progression of prostate cancer. Here we report that androgen represses the miR-99a/let7c/125b-2 cluster through AR. Androgen-activated AR binds to the host gene of the miR-99a/let7c/125b-2 cluster, LINC00478. The repression is partially mediated by the polycomb protein EZH2. Bioinformatic analysis reveals a significant enrichment of targets of miR-99a, let-7c and miR-125b in androgen-induced gene sets, suggesting that downregulation of the miR-99a/let7c/125b-2 cluster by androgen protects many of their target mRNAs from degradation and indirectly assists in the gene induction. We validated the hypothesis with twelve potential targets of the miR-99a/let7c/125b-2 cluster induced by androgen: nine out of the twelve mRNAs are downregulated by the microRNA cluster. To ascertain the biological significance of this hypothesis we focused on IGF1R, a known prostate cancer growth factor that is induced by androgen and predicted to be targeted by the miR-99a/let7c/125b-2 cluster. IGF1R is directly repressed by miR-99a and let-7c and expression of a microRNA-resistant form of IGF1R protects prostate cancer cells from inhibition by the miR-99a/let7c/125b-2 cluster. Finally, the androgen induced cell proliferation is ameliorated by preventing the repression of the microRNAs or induction of IGF1R. These results indicate that a thorough understanding of how androgen stimulates prostate cancer growth requires not only an understanding of genes directly induced/repressed by AR but also of genes indirectly induced by AR through the repression of key microRNAs.

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Since its first discovery at the end of last century, microRNAs (miRNAs) enhanced our understanding of gene regulation. These 17-25 nucleotides non-coding RNAs recognize their target mRNAs through sequence complementarity and repress target gene expression by inducing mRNA degradation or translational inhibition (1). Aberrant expression of miRNAs has been observed in many cancers and demonstrated to have central roles in tumorigenesis and malignant transformation (2-4). Prostate cancer is the second leading cause of cancer death for male in the United States. With an estimated new incidence of 241,740 and death of 28,170 in 2012, there are more than 2.5 million men have been diagnosed with prostate cancer (5). Since the invention of miRNA microarray and high throughput sequencing of small RNAs, several studies of miRNA profiling have been done in human prostate cancer specimens and cell lines. A number of miRNAs have been linked to prostate cancer from these profiling studies (3, 4, 6, 7).

In last year's annual report, we reported that miR-125b and miR-99 family of miRNAs (miR-99a, miR-99b and miR-100) were downregulated during the progression of prostate cancer using human prostate tumor specimens and two pairs of prostate cancer cell lines. Ectopic expression of miR-125b and miR-99 family inhibits prostate cancer cell proliferation. In several other independent studies of miRNA profiles, these four miRNAs were also observed to be underexpressed in prostate cancer and possessed tumor suppressive functions. We also found that miR-99a, miR-100 and miR-125b were repressed by androgen in a dose dependent manner. Interestingly, miR-99a and miR-125b-2 is colocalized with let-7c in an intron of a long non-coding RNA LINC00478, while miR-100, miR-125b-1 and let-7a-2 are localized in MIR100HG. Therefore, it is interesting to examine whether these tumor suppressive miRNAs are regulated similarly by androgen, how are they regulated as well as the biological consequences of repression of these miRNAs. We decided to follow up on these exciting questions and created an additional aim.

Supplemental aim: Determining the mechanisms and biological significance of androgen repression of miRNA clusters.

miRNA expression is tightly regulated at almost every step of its biogenesis process, which accounts for the differential expression of miRNAs during development and disease progression. However, transcriptional regulation of miRNAs is the most diverse and interesting step, as many well-known cancer-related transcription factors are shown to directly target miRNAs. Most pri-miRNAs transcribed by pol II are similarly regulated at transcriptional level as other protein coding genes. Tumor suppressor p53 is known to regulate the expression of

miRNAs that are capable to regulate cell cycle progression, apoptosis and DNA damage response, such as miR-34 family, miR-145, miR-192 and miR-215 (8-10). On the other hand, oncogene myc is known to induce the expression of oncomiR-17-92 cluster and miR-221 in various cancers (11, 12). In cancer cells, deregulation of transcription factors would therefore lead to aberrant expression of their target miRNAs, which may in turn contribute to the progression of cancers.

In the supplemental tasks, we would like to study a) whether the miR-99a/let7c/125b-2 cluster of miRNAs is repressed by androgen at transcriptional level; b) whether androgen receptor (AR) is involved in downregulation of the miR-99a/let7c/125b-2 cluster; c) whether target genes of the miR-99a/let7c/125b-2 cluster are enriched in androgen-induced genes as a result of androgen repression of miR-99a/let7c/125b-2 cluster; d) identify key targets of miR-99a/let7c/125b-2 cluster that are important for androgen stimulated growth in prostate cancer cells.

Body

Task1: Check the expression profile of AIS miRNAs.

We reported last year that the expression level of AIS miRNAs was significantly decreased in androgen independent cell line C4-2 compared to androgen dependent cell line LNCaP. It is also underexpressed in advanced prostate cancer cell line WPE1-NB26 compared to its parental prostate epithelial cells RWPE-1, as well as in prostate tumors compared to normal tissues.

This time, we also tested the expression of AIS miRNAs in another pair of androgen dependent (AD) and androgen independent (AI) cell lines, C33 and C81. Our results showed that miR-99a, miR-100 and miR-125b were also decreased in AI C81 cells compared to AD C33 cells, while miR-99b and miR-19b did not show any differences (Fig. 1). However, miR-99b showed a decrease in LAPC-4 AI xenografts compared to LAPC-4 AD xenografts which were developed in castrated and intact SCID mice respectively (Fig. 1). These results show that the decrease of AIS miRNAs in AI prostate cancer cells is a general phenomenon.

Figure 1. Expression of AIS miRNAs in prostate cancer cells.
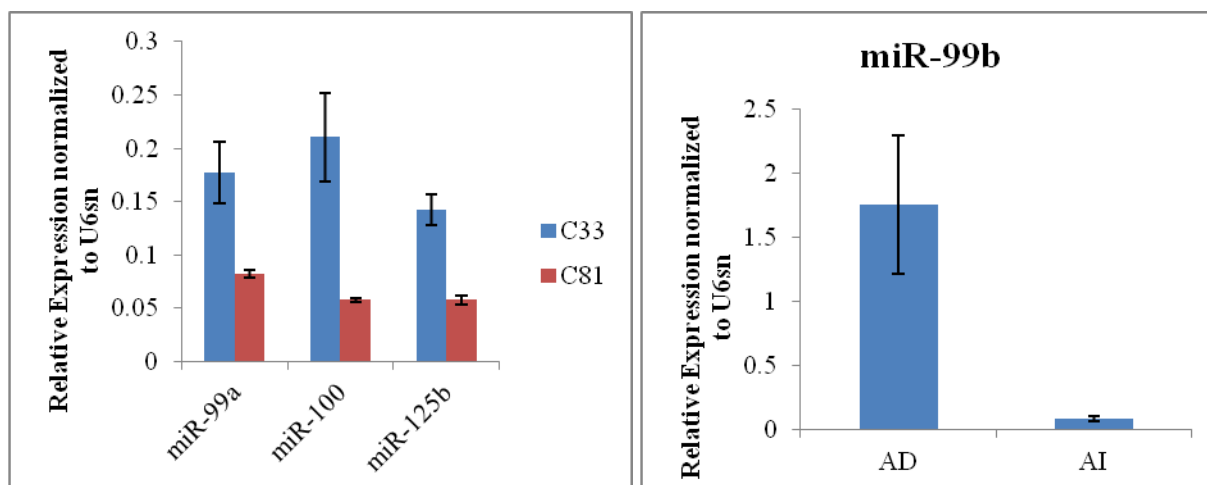
Task 2: Check if AIS miRNAs are required to maintain the androgen dependence.

We already reported last time that the reduction of miR-99 family, seen during the progression from LNCaP to C4-2, could provide a growth advantage under androgen-depleted condition. We transfected these miRNAs in C4-2, where their initial expression was low, and measured the growth of cells by BrdU incorporation assay and counting cell numbers. miR-99a, -99b or -100 inhibits the growth of C4-2 cells more markedly in the absence of androgen (CS) than in the presence of 1nM synthetic androgen R1881. This inhibition of androgen-independent growth by the miR-99 family requires the presence of AR, as the miR-99 family does not affect the growth of PC3 and Du145 cells.

This time, we also tried to inhibit AIS miRNAs by 2'-O-methyl oligonucleotides in LNCaP and measure their proliferation. However, the transient inhibition of miRNAs by 2'-O-methyl oligonucleotides was not very efficient and not enough for a long term growth assay. Other methods, such as Tough Decoy (TuD), a vector expressing antisense against target miRNA, may be considered to achieve this goal.

Task 3: Examine the histone modification of AIS miRNA genes.

We found that AIS miRNAs miR-99a, miR-100 and miR-125b were repressed by androgen in a dose dependent manner. Interestingly, miR-99a and miR-125b-2 is colocalized with let-7c in an intron of a long non-coding RNA LINC00478, while miR-100, miR-125b-1 and let-7a-2 are localized in MIR100HG. To distinguish between the clusters, we checked whether let-7c or let-7a was repressed by androgen in a manner similar to miR-99a, miR-100 and miR-125b. let-7c, but not let-7a, was repressed by synthetic androgen R1881 in a dose dependent

manner in LNCaP cells, suggesting that the miR-99a/let7c/125b-2 cluster is responsive to androgen (Fig. 2A). To test whether androgen represses the transcription of the cluster, we measured the expression of primary miRNAs of the miR-99a/let7c/125b-2 cluster and its host gene LINC00478. Pri-miR-99a, pri-let7c, pri-miR-125b-1 and LINC00478 were all decreased by androgen (Fig. 2).

Figure 2. miR-99a/let7c/125b-2 cluster and its host gene is repressed by androgen.
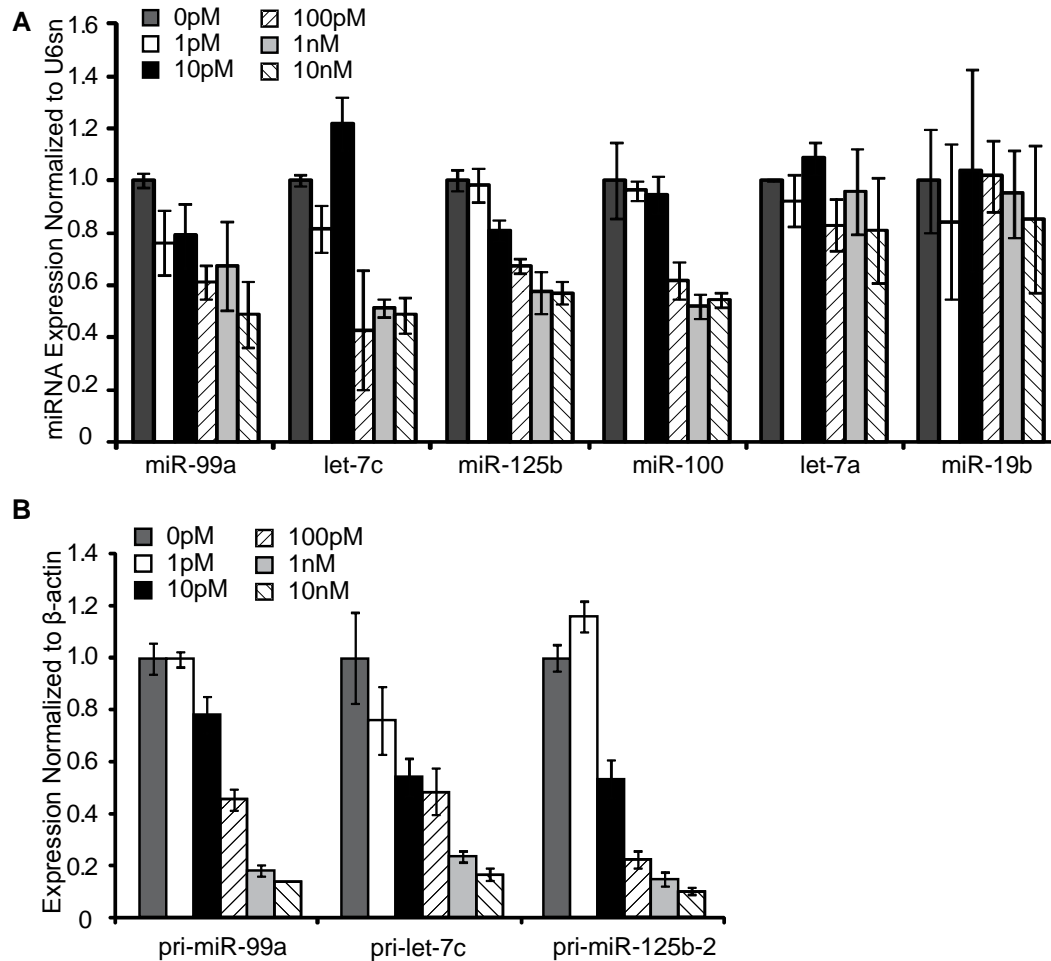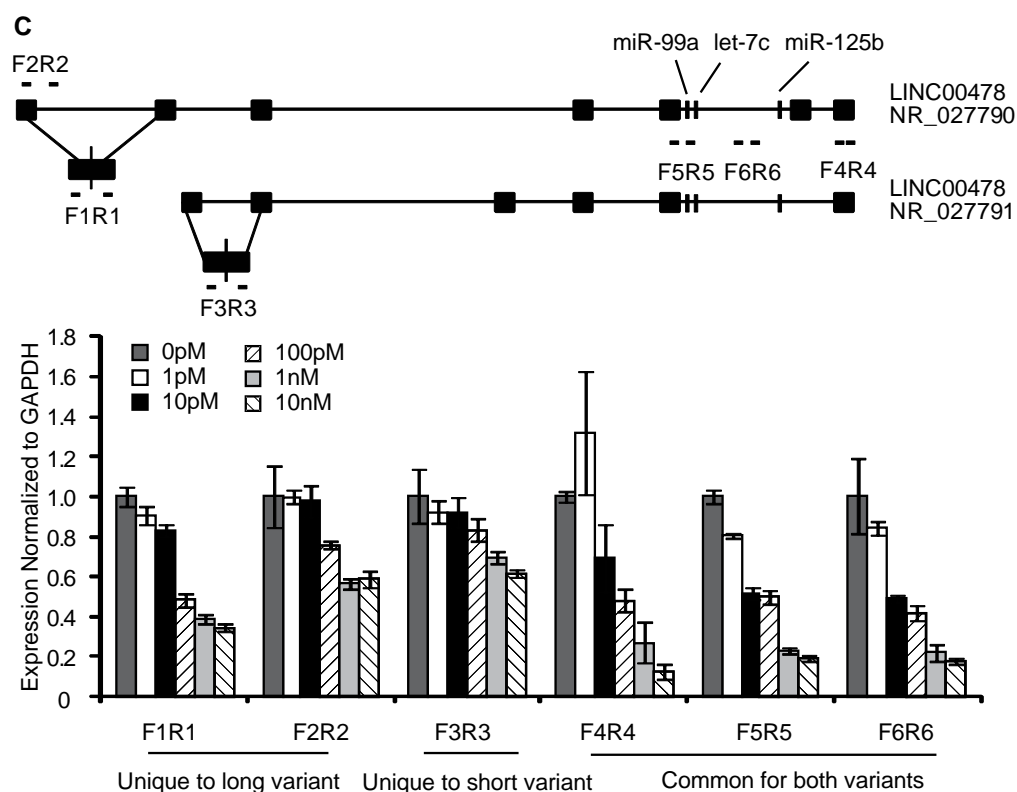
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It has previously been shown that androgen-induced gene expression is mediated by forkhead box protein A1 (FOXA1) and active histone modifications including H3K4 me1 and H3K4me2. However, the mechanisms of androgen-mediated gene repression are more complex. Global ChIP-seq analysis revealed EZH2 binding and a significant enrichment of repressive histone mark H3K27me3 around AR-repressed genes, which suggested that EZH2 was involved in AR-repressed gene expression. To test whether EZH2 was responsible for the downregulation of the miR-99a/let7c/125b-2 cluster by androgen treatment, we transfected siRNA against EZH2 in LNCaP cells, and examined the expression of the miR-99a/let7c/125b-2 cluster in the absence or the presence of androgen. Depletion of EZH2 significantly diminished the repression of the miR-99a/let7c/125b-2 cluster by androgen (Fig. 3). This suggested that EZH2 and its regulated H3K27 methylation were required for androgen repression of miR-99a/let7c/125b-2 cluster.

Figure 3. Histone H3K27 methyltransferase EZH2 is required for androgen repression of miR-99a/let7c/125b-2 cluster.
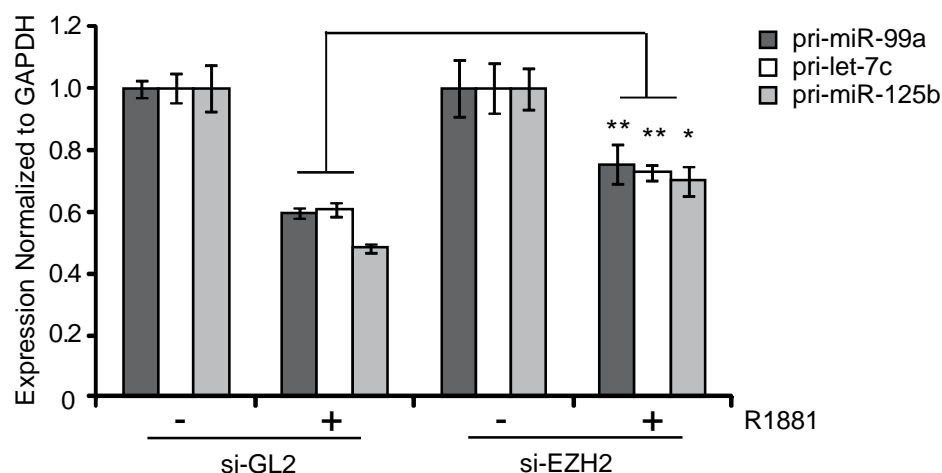
Task 4: Identify target genes of AIS miRNAs.

miRNAs and their targets form a complex gene regulatory network: one miRNA could target several genes at the same time and several miRNAs could also coordinately regulate a specific target in cancer cells. Last time we identified several targets that were directly regulated by miR-99 family by using bioinformatic approach, mRNA degradation and translational inhibition analyses. This time, we focused on indentifying targets that are synergistically regulated by miR-99a/let7c/125b-2 cluster. Using bioinformatic prediction algorithm TargetScan, we identified 7 genes that are commonly targeted by miR-99a, let-7c and miR-125b. These genes are DOCK3, PLAGL2, PRDM1, RASL10B, SLC4A4, TRIB2 and IGF1R (Table 1).

Table 1. Predicted genes that are commonly targeted by miR-99a, let-7c and miR-125b.

Gene	Description	Note
DOCK3	dedicator of cytokinesis 3	enhances cell adhesion and inhibits Wnt signaling
PLAGL2	pleiomorphic adenoma gene-like 2	an oncogene involved in various malignancies including lipoblastomas, hepatoblastomas, and acute myeloid leukemia. PLAGL2 can bind and activate human insulin-like growth factor II (IGF II) gene promoter. PLAGL2 controls the stability of Pirh2, an E3 ubiquitin ligase for p53. Tip60 modulates PLAGL2-mediated transactivation by acetylation.
PRDM1	PR domain containing 1, with ZNF domain	Transcriptional repressor that binds specifically to the PRDI element in the promoter of the beta-interferon gene.
RASL10B	RAS-like, family 10, member B	Belongs to the small GTPase superfamily, Ras family, downregulated in breast tumor

SLC4A4	solute carrier family 4, sodium bicarbonate cotransporter, member 4	a sodium bicarbonate cotransporter (NBC) involved in the regulation of bicarbonate secretion and absorption and intracellular pH.
TRIB2	tribbles homolog 2 (Drosophila)	A protein kinase that controls MAPK cascades. Oncogene Trib2 and HoxA9 cooperate to accelerate acute myelogenous leukemia. TRIB2 is a repressor of FOXO that contributes to the malignant phenotype of melanoma cells.
IGF1R	Insulin-like growth factor 1 receptor	Oncogene that is involved in angiogenesis, transformation and mitogenesis of prostate cancer. It is frequently overexpressed in prostate tumors and often associated with poor prognosis. Several monoclonal antibodies and small molecule inhibitors of IGF1R are currently under investigation in clinical trials for treating metastatic castration-resistant prostate cancer.

Task 5: Test a few predicted targets of AIS miRNAs to confirm the true targets.

Among the 7 genes predicted to be regulated by all three miRNAs of miR-99a/let7c/125b-2 cluster at the same time, we were particularly interested in IGF1R and TRIB2. TRIB2, a protein kinase that controls mitogen-activated protein kinase cascades, is reported as an oncogene, as TRIB2 and HoxA9 cooperate to accelerate acute myelogenous leukemia. It is also a repressor of FOXO that contributes to the malignant phenotype of melanoma cells. However, luciferase reporter assay of TRIB2 3'UTR showed that only miR-99a directly binds to TRIB2 but not let-7c or miR-125b (Fig. 4).

Figure 4. Luciferase Reporter Assay of TRIB2 3'UTR.
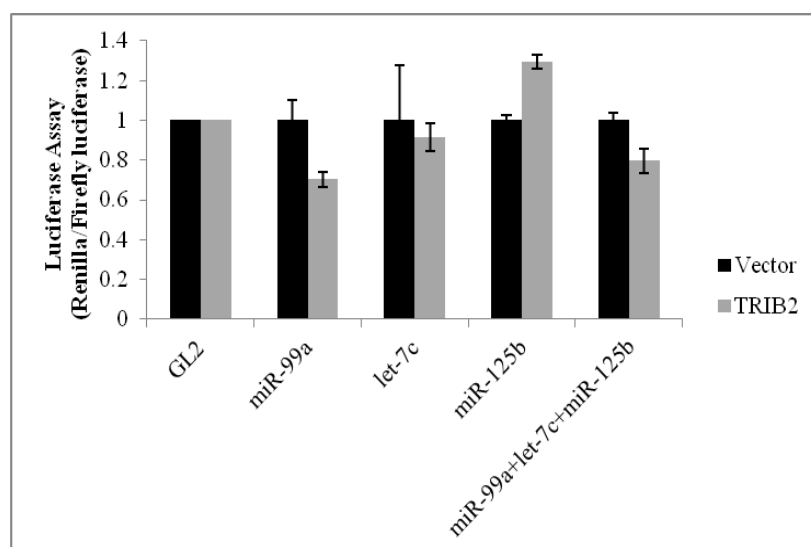
IGF1R is important for the tumorigenesis and progression of prostate cancer, because of its demonstrated roles in angiogenesis, transformation and mitogenesis (13-15). It is frequently

overexpressed in prostate tumors and often associated with poor prognosis (16, 17). Several monoclonal antibodies and small molecule inhibitors of IGF1R are currently under investigation in clinical trials for treating metastatic castration-resistant prostate cancer (CRPC) (18, 19). More importantly, the expression of IGF1R is increased by androgen, consistent with androgen repression of miR-99a/let7c/125b-2 cluster. In the luciferase reporter assay, ectopic expression of miR-99a and let-7c but not miR-125b decreased the luciferase activity, indicating that miR-99a and let-7c directly bound to the 3'UTR of IGF1R and regulated its expression (Fig 5A). Furthermore, overexpression of the miR-99a/let7c/125b-2 cluster decreased both mRNA and protein level of IGF1R in LNCaP cells (Fig. 5B&C). These data suggest that miR-99a and let-7c from miR-99a/let7c/125b-2 cluster target IGF1R together.

Figure 5. IGF1R is a direct target of miR-99a/let7c/125b-2 cluster.
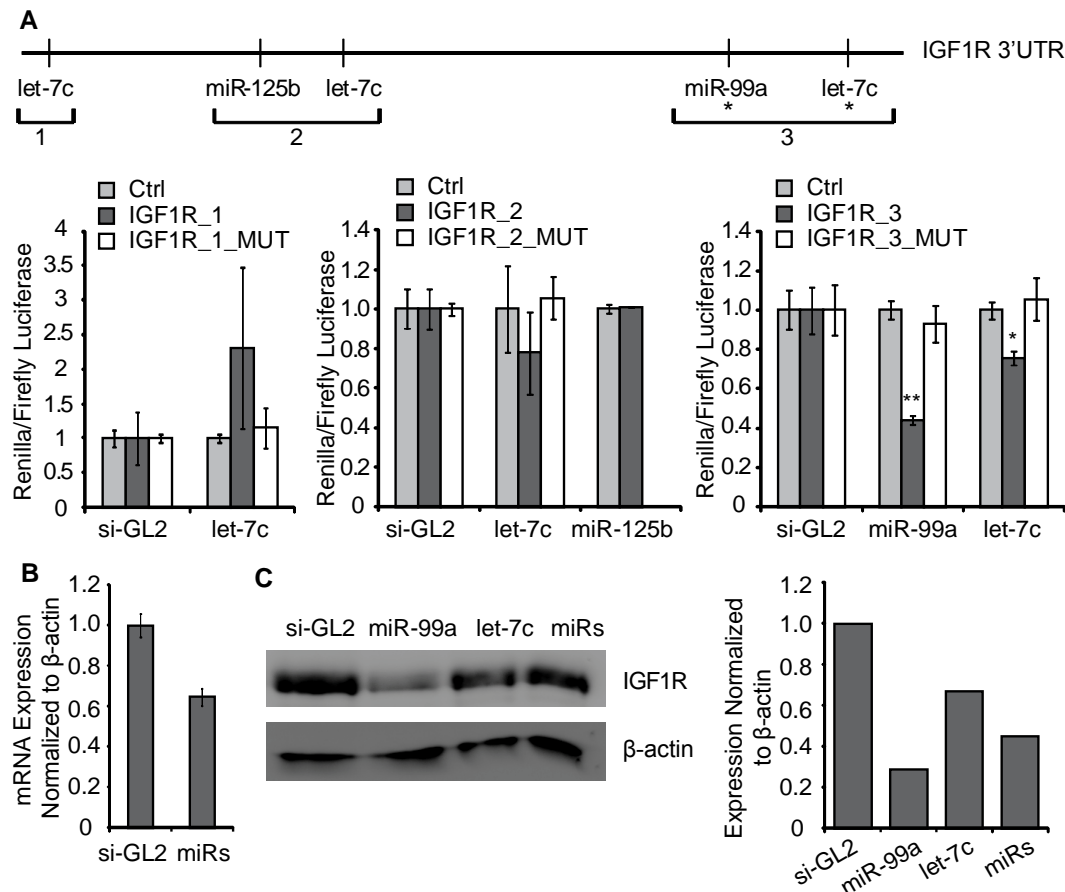
Androgen is known to be capable of stimulating the growth of prostate cancer cells, and members of the miR-99a/let7c/125b-2 cluster have been shown previously to have growth suppressive functions (3, 20-23). We therefore hypothesized that androgen-repression of the

miR-99a/let7c/125b-2 cluster promoted androgen-dependent growth through the derepression of IGF1R. To test this hypothesis, we first performed a growth assay in LNCaP cells. Addition of androgen stimulated proliferation of LNCaP cells (Fig. 6, CS v.s. 1nM R1881 si-GL2), while forced expression of miR-99a, let-7c or miR-125b to prevent their downregulation by androgen blunted the growth stimulatory effect of androgen (Fig. 6). siRNA against IGF1R generated a similar phenotype (Fig. 6), suggesting that miR-99a and let-7c, the two miRNAs we found to directly regulate IGF1R expression, may inhibit LNCaP cell growth through targeting IGF1R. To test this possibility, we generated a LNCaP cell line that stably expresses IGF1R, which lacks the 3'UTR and is not regulated by miRNAs. Overexpression of IGF1R without its 3'UTR rescued the growth defect caused by miR-99a, let-7c or siRNA targeting the 3'UTR of IGF1R, indicating that the growth suppression of LNCaP cells by the miR-99a/let7c/125b-2 cluster in the presence of androgen was primarily due to the downregulation of IGF1R (Fig. 6).   The result also suggests that repression of the miR-99a/let7c/125b-2 cluster by androgen will contribute to prostate cancer growth by relieving IGF1R from microRNA inhibition.

Figure 6. IGF1R is the key target that is responsible for the growth inhibition by miR-99a/let7c/125b-2 cluster,
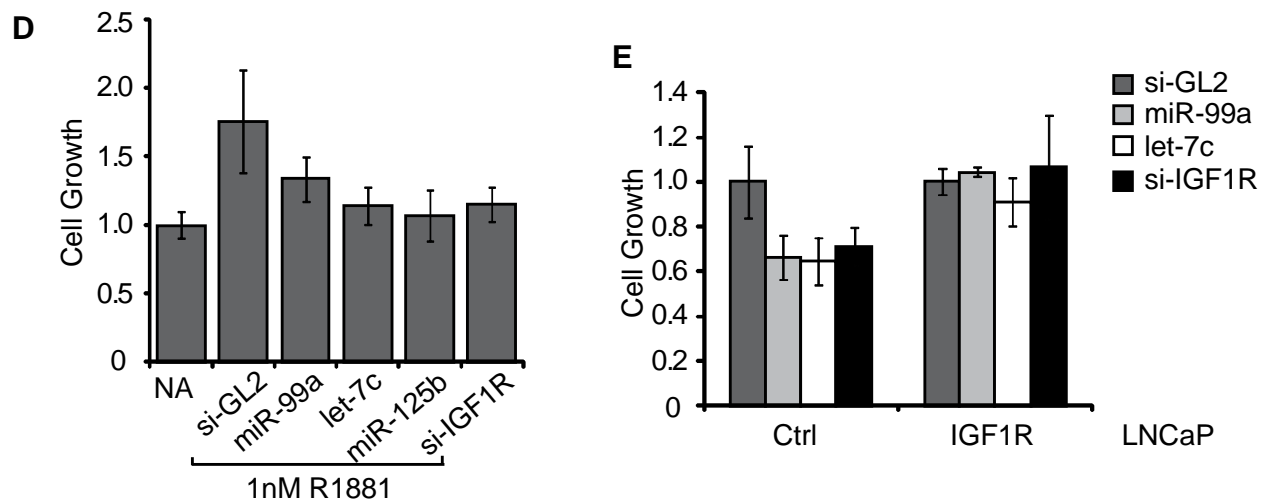
Supplementary Tasks: (See Appendix. Figure and Table numbers mentioned in this section are referring to those in appendix too.)

   a. **Check whether the miR-99a/let7c/125b-2 cluster of miRNAs is repressed by androgen at transcriptional level**

miR-99a, miR-100 and miR-125b are downregulated by androgen treatment (3), but the mechanism of downregulation was not known. The two genomic loci of miR-125b, miR-125b-1 on chromosome 11 is encoded in mir-100/let-7a-2 cluster (MIR100HG) while miR-125b-2 on chromosome 21 is encoded with miR-99a and let-7c within an intron of a long non-coding RNA LINC00478 (Table 1 and Fig. 1C), while miR-125b-1 (Table 1). To distinguish between the clusters, we checked whether let-7c or let-7a was repressed by androgen in a manner similar to miR-99a, miR-100 and miR-125b. let-7c, but nor let-7a, was repressed by synthetic androgen R1881 in a dose dependent manner in LNCaP cells (Fig. 1A), suggesting that the miR-99a/let7c/125b-2 cluster is responsive to androgen. To test whether androgen represses the transcription of the cluster, we measured the expression of primary miRNAs of the miR-99a/let7c/125b-2 cluster. Pri-miR-99a, pri-let7c and pri-miR-125b-1 were all decreased by androgen (Fig. 1B).

Since the miR-99a/let7c/125b-2 cluster is located in an intron , we examined whether the host gene, LINC00478, is also repressed by androgen. Two transcripts of LINC00478, long (NR_027790) and short (NR_027791), could encode the miR-99a/let7c/125b-2 cluster (Fig. 1C). Six pairs of primers were used to assess androgen regulation of both transcripts (Fig. 1C). F1R1 and F2R2 detect the mature and precursor forms of the long transcript, respectively. F3R3 detects the mature short transcript, while the other three primer pairs detect both transcripts. Both transcripts of LINC00478 were downregulated by androgen, with the long transcript more responsive than the short transcript (Fig. 1C; F1R1 vs. F3R3).

b. **Determine whether androgen receptor (AR) is involved in downregulation of the miR-99a/let7c/125b-2 cluster**

We next determine whether AR is required for the androgen-repression of the miR-99a/let7c/125b-2 cluster. As in the AR-positive LNCaP cells, the expression of the miR-99a/let7c/125b-2 cluster was decreased by androgen in AR-positive C4-2 prostate cancer cells (Fig. 2A and Fig. S1A), but not in AR-negative PC-3 prostate cancer cells (Fig. 2B and Fig. S1B). PC-3 cells have a wild type AR gene but do not express AR due to weak transcriptional activity at the AR promoter (24). Ectopic expression of wild type AR in PC-3 cells (Fig. S2B) restored the repression of miR-99a/let7c/125b-2 by androgen

(Fig. 2C and Fig. S1C). Conversely, knocking down AR in LNCaP cells by siRNA (Fig. S2A) abolished the repression of miR-99a/let7c/125b-2 by androgen (Fig. 2D and Fig. S1D). These results suggest that AR is required for androgen to repress miR-99a/let7c/125b-2 in prostate cancer cells.

Next we evaluated whether AR directly binds to the host gene of the miR-99a/let7c/125b-2 cluster, LINC00478. AR ChIP-seq data in LNCaP cells in the presence or absence of R1881 have been published (25). Peaks of AR binding were identified using HPeak, a Hidden Markov Model (HMM)-based peak identifying algorithm (http://www.sph.umich.edu/csg/qin/HPeak/). Seven AR binding sites were reported by the ChIP-seq upstream and within the LINC00478 gene: ARBS1-7 (Fig. 3A). ARBS1 and ARBS2 are within 50kb of the transcription start site (TSS) of the long transcript and ARBS3, ARBS4 and ARBS5 are within 50kb of the TSS of the short transcript. Since genome-wide ChIP-seq analyses often have significant false-positive rates, AR ChIP and quantitative real-time PCR was performed to validate whether AR bound to any of these five potential binding sites. Androgen induced AR binding to ARBS1 and ARBS2 (Fig. 3B) to an extent that was similar to the binding to the well-characterized PSA enhancer (AREIII) (26). Significant AR binding or AREs were not seen at the other AR ChIP-seq sites. Interestingly, ARE half-sites were detected at ARBS1 and ARBS2 by ALGGEN-PROMO, a matrix algorithm for predicting transcription factor binding sites based on TRANSFAC (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3) (Fig. S2D). ARBS1 contains "TGTCCT", an exact half site of canonical ARE (27), while ARBS2 contains "GGGACA", which resembles the other half of the canonical ARE ("GGTACA" or "GGAACA") (27).

c. **Examine whether target genes of the miR-99a/let7c/125b-2 cluster are enriched in androgen-induced genes as a result of androgen repression of miR-99a/let7c/125b-2 cluster**

Since the miR-99a/let7c/125b-2 cluster was repressed by androgen, we hypothesized that target genes of miR-99a, let-7c and miR-125b may be upregulated upon androgen treatment as a result of reduced targeting by the miR-99a/let7c/125b-2 cluster. To test this hypothesis, we extracted lists of potential targets of miR-99a, let-7c and miR-

125b from TargetScan (version 6.0), a widely used target prediction algorithm of miRNAs. Two lists of androgen-induced genes were obtained from two independent studies (GSE17044 and GSE22606), in which LNCaP cells were treated with R1881or vehicle and gene expression was measured by microarray analysis. To determine which (and how many) androgen-induced genes were also miRNA targets, we intersected predicted targets of each miRNA with androgen-induced genes from each microarray dataset (Table S1, S2 and S3). We performed a Monte Carlo simulation to determine the expected number of miRNA targets in the androgen-induced gene sets at random. The fold enrichment (with p-value) was calculated of the observed number of androgen-induced miRNA targets relative to the random expectation. Potential targets of miR-99a were 4.92 and 6.63 fold enriched in the two respective androgen-induced gene sets, while targets of let-7c and miR-125b were also significantly enriched in androgen-upregulated genes (Table 2).

Table 3 contains the androgen-induced genes that are targets of the miR-99a/let7c/125b-2 cluster that were common between the two microarray datasets used. We selected four target genes of each miRNA and validated by qRT-PCR that they were induced after androgen treatment. The expression of all of the genes we tested was increased by androgen (Fig. 4A). To test whether the AR-induced genes were also repressed by the miR-99a/let7c/125b-2 cluster, we measured their mRNAs by qRT-PCR after miRNA transfection. At least 75% of the mRNAs tested were significantly decreased by the miR-99a/let7c/125b-2 cluster (Fig. 4B). Since the androgen-repression of the miR-99a/let7c/125b-2 cluster requires AR, we also examined the expression of these mRNAs after knocking-down AR by siRNA. None of the selected genes were stimulated by androgen after AR knock-down (Fig. S3A, B and C). Thus, many genes induced by androgens through AR, are concurrently de-repressed through AR mediated inhibition of the miR-99a/let7c/125b-2 cluster.

d. **Identify key targets of miR-99a/let7c/125b-2 cluster that are important for androgen stimulated growth in prostate cancer cells.**

Among the androgen-induced target genes of the miR-99a/let7c/125b-2 cluster, factors in the IGF signaling pathway appeared multiple times: insulin-like growth factor 1 receptor (IGF1R) was a target of miR-99a and let-7c, and insulin receptor substrate 2

(IRS2) was a target of let-7c. miR-125b was also predicted to target IGF1R, but at a site that is conserved only among primates. IGF1R is important for the tumorigenesis and progression of prostate cancer, because of its demonstrated roles in angiogenesis, transformation and mitogenesis (13-15). It is frequently overexpressed in prostate tumors and often associated with poor prognosis (16, 17). Several monoclonal antibodies and small molecule inhibitors of IGF1R are currently under investigation in clinical trials for treating metastatic castration-resistant prostate cancer (CRPC) (18, 19). We therefore decided to follow up on IGF1R and first validated that IGF1R was a direct target of the miR-99a/let7c/125b-2 cluster by luciferase reporter assay (Fig. 5A). In the 3'UTR of IGF1R there was one predicted binding site each for miR-99a and miR-125b and three for let-7c (Fig. 5A). It was shown previously that let-7 directly downregulated several genes in the IGF signaling pathway, including IGF1R, but the report did not clarify whether all three target sites were active in IGF1R repression (28). Therefore, we tested the effect of each miRNA target site on IGF1R by dividing the 3'UTR of IGF1R into three fragments and cloning each of them into a luciferase reporter. The first fragment contained one let-7c binding site, the second fragment had the miR-125b binding site and another let-7c binding site, and the third fragment included the miR-99a binding site and the third let-7c binding site (Fig. 5A). In the luciferase reporter assay, ectopic expression of miR-99a and let-7c but not miR-125b decreased the luciferase activity, indicating that miR-99a and let-7c directly bound to the 3'UTR of IGF1R and regulated its expression (Fig. 5A). The two 3'-most binding sites of let-7c responded to the microRNA, with the most significant regulation coming from the third binding site. The differential effects of these miRNA binding sites might be due to the secondary structure of the target mRNA or the binding of other proteins to the target mRNA.

We then checked the expression level of IGF1R after transfecting the miR-99a/let7c/125b-2 cluster into cells. Overexpression of the miR-99a/let7c/125b-2 cluster decreased both mRNA and protein level of IGF1R in LNCaP cells (Fig. 5B and C).

Androgen is known to be capable of stimulating the growth of prostate cancer cells, and members of the miR-99a/let7c/125b-2 cluster have been shown previously to have growth suppressive functions (3, 20-23). We therefore hypothesized that androgen-repression of the miR-99a/let7c/125b-2 cluster promoted androgen-dependent growth through the derepression of IGF1R. To test this hypothesis, we first performed a growth

assay in LNCaP cells. Addition of androgen stimulated proliferation of LNCaP cells (Fig. 5D, CS v.s. 1nM R1881 si-GL2), while forced expression of miR-99a, let-7c or miR-125b to prevent their downregulation by androgen blunted the growth stimulatory effect of androgen (Fig. 5D). siRNA against IGF1R generated a similar phenotype (Fig. 5D), suggesting that miR-99a and let-7c, the two miRNAs we found to directly regulate IGF1R expression, may inhibit LNCaP cell growth through targeting IGF1R. To test this possibility, we generated a LNCaP cell line that stably expresses IGF1R, which lacks the 3'UTR and is not regulated by miRNAs (Fig. S2C). Overexpression of IGF1R without its 3'UTR rescued the growth defect caused by miR-99a, let-7c or siRNA targeting the 3'UTR of IGF1R, indicating that the growth suppression of LNCaP cells by the miR-99a/let7c/125b-2 cluster in the presence of androgen was primarily due to the downregulation of IGF1R (Fig. 5E).   The result also suggests that repression of the miR-99a/let7c/125b-2 cluster by androgen will contribute to prostate cancer growth by relieving IGF1R from microRNA inhibition (Fig. 5F).

Key Research Accomplishments

1. Discovered an androgen-repressed miRNA cluster miR-99a/let-7c/miR-125b-2.
2. Determined the mechanism of androgen repression: androgen-activated androgen receptor binds directly to the enhancer of the host gene of miR-99a/let-7c/miR-125b-2, LINC00478, recruits polycomb protein EZH2 and inhibits their transcription. This is the first report that elucidates the mechanisms of androgen repression of miRNAs.
3. Showed the tumor suppressive function of miR-99a/let-7c/miR-125b-2 cluster in prostate cancer cells.
4. Demonstrated an alternate way of gene regulation by androgen through miRNAs.
5. Identified IGF1R as a target of miR-99a/let-7c/miR-125b-2 cluster.
6. Demonstrated that the growth suppression of prostate cancer cells by miR-99a/let-7c/miR-125b-2 cluster in the presence of androgen is mainly though downregulating IGF1R.

Reportable Outcomes

miR-99 family of microRNAs suppresses the expression of prostate specific antigen and prostate cancer cell proliferation

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Androgen Represses Expression of a miRNA Cluster through AR

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Regulation of several androgen-induced genes through the repression of the miR-99a/let-7c/miR-125b-2 miRNA cluster in prostate cancer cells

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Conclusions

We discovered the downregulation of the miR-99a/let7c/125b-2 cluster by androgen in prostate cancer cells. The miR-99a/let7c/125b-2 cluster and its host gene LINC00478 are transcriptionally repressed by androgen-activated AR. The repression is partially mediated by the polycomb protein EZH2. Bioinformatic analysis reveals a significant enrichment of targets of miR-99a, let-7c and miR-125b in androgen-induced gene sets, suggesting that downregulation of the miR-99a/let7c/125b-2 cluster by androgen protects many of their target mRNAs from degradation and indirectly assists in the gene induction. IGF1R is one of the androgen-induced targets of miR-99a/let7c/125b-2 cluster. We found that the androgen induced cell proliferation is abrogated by preventing the repression of the microRNAs, while overexpression of IGF1R impaired this process, suggesting that IGF1R is the key target that mediated growth inhibition by miR-99a/let7c/125b-2 cluster in prostate cancer cells.

References

1.   Kim VN. MicroRNA biogenesis: coordinated cropping and dicing. Nat Rev Mol Cell Biol 2005;6(5):376-85.
2.   Lu J, Getz G, Miska EA, Alvarez-Saavedra E, Lamb J, Peck D, et al. MicroRNA expression profiles classify human cancers. Nature 2005;435(7043):834-8.
3.   Sun D, Lee YS, Malhotra A, Kim HK, Matecic M, Evans C, et al. miR-99 family of MicroRNAs suppresses the expression of prostate-specific antigen and prostate cancer cell proliferation. Cancer Res 2011;71(4):1313-24.
4.   Jalava SE, Urbanucci A, Latonen L, Waltering KK, Sahu B, Janne OA, et al. Androgen-regulated miR-32 targets BTG2 and is overexpressed in castration-resistant prostate cancer. Oncogene 2012.
5.   http://seer.cancer.gov/statfacts/html/prost.html.

6.   Sun T, Yang M, Chen S, Balk S, Pomerantz M, Hsieh CL, et al. The altered expression of MiR-221/-222 and MiR-23b/-27b is associated with the development of human castration resistant prostate cancer. Prostate 2012;72(10):1093-103.

7.   Shi GH, Ye DW, Yao XD, Zhang SL, Dai B, Zhang HL, et al. Involvement of microRNA-21 in mediating chemo-resistance to docetaxel in androgen-independent prostate cancer PC3 cells. Acta Pharmacol Sin 2010;31(7):867-73.

8.   Suzuki HI, Miyazono K. Dynamics of microRNA biogenesis: crosstalk between p53 network and microRNA processing pathway. J Mol Med (Berl) 2010;88(11):1085-94.

9.   Jain AK, Allton K, Iacovino M, Mahen E, Milczarek RJ, Zwaka TP, et al. p53 regulates cell cycle and microRNAs to promote differentiation of human embryonic stem cells. PLoS Biol 2012;10(2):e1001268.

10.   Georges SA, Biery MC, Kim SY, Schelter JM, Guo J, Chang AN, et al. Coordinated regulation of cell cycle transcripts by p53-Inducible microRNAs, miR-192 and miR-215. Cancer Res 2008;68(24):10105-12.

11.   O'Donnell KA, Wentzel EA, Zeller KI, Dang CV, Mendell JT. c-Myc-regulated microRNAs modulate E2F1 expression. Nature 2005;435(7043):839-43.

12.   Schulte JH, Horn S, Otto T, Samans B, Heukamp LC, Eilers UC, et al. MYCN regulates oncogenic MicroRNAs in neuroblastoma. Int J Cancer 2008;122(3):699-704.

13.   Kojima S, Inahara M, Suzuki H, Ichikawa T, Furuya Y. Implications of insulin-like growth factor-I for prostate cancer therapies. Int J Urol 2009;16(2):161-7.

14.   Papatsoris AG, Karamouzis MV, Papavassiliou AG. Novel insights into the implication of the IGF-1 network in prostate cancer. Trends Mol Med 2005;11(2):52-5.

15.   Ozkan EE. Plasma and tissue insulin-like growth factor-I receptor (IGF-IR) as a prognostic marker for prostate cancer and anti-IGF-IR agents as novel therapeutic strategy for refractory cases: a review. Mol Cell Endocrinol 2011;344(1-2):1-24.

16.   Cardillo MR, Monti S, Di Silverio F, Gentile V, Sciarra F, Toscano V. Insulin-like growth factor (IGF)-I, IGF-II and IGF type I receptor (IGFR-I) expression in prostatic cancer. Anticancer Res 2003;23(5A):3825-35.

17.   Liao Y, Abel U, Grobholz R, Hermani A, Trojan L, Angel P, et al. Up-regulation of insulin-like growth factor axis components in human primary prostate cancer correlates with tumor grade. Hum Pathol 2005;36(11):1186-96.

18.   Gennigens C, Menetrier-Caux C, Droz JP. Insulin-Like Growth Factor (IGF) family and prostate cancer. Crit Rev Oncol Hematol 2006;58(2):124-45.

19.   Antonarakis ES, Carducci MA, Eisenberger MA. Novel targeted therapeutics for metastatic castration-resistant prostate cancer. Cancer Lett 2010;291(1):1-13.

20.   Guan Y, Yao H, Zheng Z, Qiu G, Sun K. MiR-125b targets BCL3 and suppresses ovarian cancer proliferation. Int J Cancer 2011;128(10):2274-83.

21.   Nadiminty N, Tummala R, Lou W, Zhu Y, Shi XB, Zou JX, et al. MicroRNA let-7c is downregulated in prostate cancer and suppresses prostate cancer growth. PLoS One 2012;7(3):e32832.

22.   Huang L, Luo J, Cai Q, Pan Q, Zeng H, Guo Z, et al. MicroRNA-125b suppresses the development of bladder cancer by targeting E2F3. Int J Cancer 2011;128(8):1758-69.

23.   Mueller AC, Sun D, Dutta A. The miR-99 family regulates the DNA damage response through its target SNF2H. Oncogene 2012.

24.   Chlenski A, Nakashiro K, Ketels KV, Korovaitseva GI, Oyasu R. Androgen receptor expression in androgen-independent prostate cancer cell lines. Prostate 2001;47(1):66-75.

25.   Yu J, Mani RS, Cao Q, Brenner CJ, Cao X, Wang X, et al. An integrated network of androgen receptor, polycomb, and TMPRSS2-ERG gene fusions in prostate cancer progression. Cancer Cell 2010;17(5):443-54.

26.   Cai C, He HH, Chen S, Coleman I, Wang H, Fang Z, et al. Androgen receptor gene expression in prostate cancer is directly suppressed by the androgen receptor through recruitment of lysine-specific demethylase 1. Cancer Cell 2011;20(4):457-71.

27.   Takayama K, Kaneshiro K, Tsutsumi S, Horie-Inoue K, Ikeda K, Urano T, et al. Identification of novel androgen response genes in prostate cancer cells by coupling chromatin immunoprecipitation and genomic microarray analysis. Oncogene 2007;26(30):4453-63.

28.   Zhu H, Shyh-Chang N, Segre AV, Shinoda G, Shah SP, Einhorn WS, et al. The Lin28/let-7 axis regulates glucose metabolism. Cell 2011;147(1):81-94.

Appendix

Regulation of several androgen-induced genes through the repression of the miR-99a/let-7c/miR-125b-2 miRNA cluster in prostate cancer cells

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Running Title: androgen-repressed miRNA cluster

Abstract

The androgen receptor (AR) stimulates and represses gene expression to promote the initiation and progression of prostate cancer. Here we report that androgen represses the miR-99a/let7c/125b-2 cluster through AR. Androgen-activated AR binds to the host gene of the miR-99a/let7c/125b-2 cluster, LINC00478. The repression is partially mediated by the polycomb protein EZH2. Bioinformatic analysis reveals a significant enrichment of targets of miR-99a, let-7c and miR-125b in androgen-induced gene sets, suggesting that downregulation of the miR-99a/let7c/125b-2 cluster by androgen protects many of their target mRNAs from degradation and indirectly assists in the gene induction. We validated the hypothesis with twelve potential targets of the miR-99a/let7c/125b-2 cluster induced by androgen: nine out of the twelve mRNAs are downregulated by the microRNA cluster. To ascertain the biological significance of this hypothesis we focused on IGF1R, a known prostate cancer growth factor that is induced by androgen and predicted to be targeted by the miR-99a/let7c/125b-2 cluster. IGF1R is directly repressed by miR-99a and let-7c and expression of a microRNA-resistant form of IGF1R protects prostate cancer cells from inhibition by the miR-99a/let7c/125b-2 cluster. Finally, the androgen induced cell proliferation is ameliorated by preventing the repression of the microRNAs or induction of IGF1R. These results indicate that a thorough understanding of how androgen stimulates prostate cancer growth requires not only an understanding of genes directly induced/repressed by AR but also of genes indirectly induced by AR through the repression of key microRNAs.

Introduction

　　The androgen receptor (AR) is essential for normal prostate development as well as the

progression of prostate cancer. AR is typically activated by ligand-binding and translocates to the

nucleus where it binds to the androgen responsive elements (AREs) and transactivates gene

expression (1). The canonical ARE consists of an inverted repeat sequence 5'-

AGAACAnnnTGTTCT-3' (http://transfac.gbf.de/TRANSFAC/) (2, 3). Genome-wide studies

have revealed other non-canonical AREs sequences and ARE half-sites (4-7). Recent studies

reported that androgen represses as many genes as it induces, and the majority of androgen-

repressed genes bind AR nearby (8, 9). Androgen-activated AR actively represses gene

expression, including the AR gene itself (10-13). Androgen-bound AR could repress genes by

directly binding to activating transcription factors utilized by the promoter or competing for

cofactors and thus interfering with its transcriptional activity (14-16). AR can also recruit

corepressor complexes and histone modification enzymes (9-11, 13). AR-repressed genes

displayed similar classification and distribution in gene ontology and pathway analyses as

androgen-induced genes and the repression contributes to prostate cancer cell growth, survival

and invasion (8, 11, 16).

　　miRNAs belong to a class of small non-coding RNA that regulates gene expression

posttranscriptionally. Their aberrant expression has been observed in various diseases, including

prostate cancer (17-20). Several groups have reported regulation of miRNAs by androgen with a

focus on the miRNAs upregulated by androgen. miR-32 and miR-148a were upregulated by

androgen and overexpressed in prostate cancers compared to benign prostate hyperplasia (21).

miR-29a, miR-29b and miR-141 were also induced by androgen in prostate cancer cells (22).

miR-22 and miR-134 are upregulated and miR-17, miR-18 and miR-20a are downregulated by

androgens (23). miRNAs can be regulated at the levels of transcription, biogenesis or degradation

(24). miR-21 was transcriptionally induced by AR directly bound to the miR-21 gene (25). On

the other hand, androgen not only increased the transcription of the miR-23a/27a/24-2 cluster,

but also promoted Drosha-mediated primary miRNA processing (26). Regulation of miRNA

expression by androgen results in aberrant expression of miRNA target genes, which in turn may

affect the progression of prostate cancer.

In this study, we report the downregulation of the miR-99a/let7c/125b-2 cluster by

androgen in prostate cancer cells. The miR-99a/let7c/125b-2 cluster is transcriptionally

repressed by androgen-activated AR. There is a significant enrichment of target genes of the

miR-99a/let7c/125b-2 cluster among androgen-induced genes, suggesting that gene induction by

androgen in prostate cancer is indirectly assisted through repression of specific miRNAs. The

importance of this indirect regulation of gene expression by AR is revealed by the abrogation of

androgen-stimulated cell proliferation when the decrease of mR-99a/let7c/125b-2 is prevented.

Results

The miR-99a/let7c/125b-2 cluster of miRNAs is repressed by androgen

miR-99a, miR-100 and miR-125b are downregulated by androgen treatment (19), but the

mechanism of downregulation was not known. The two genomic loci of miR-125b, miR-125b-1

on chromosome 11 is encoded in mir-100/let-7a-2 cluster (MIR100HG) while miR-125b-2 on

chromosome 21 is encoded with miR-99a and let-7c within an intron of a long non-coding RNA

LINC00478 (Table 1 and Fig. 1C), while miR-125b-1 (Table 1). To distinguish between the

clusters, we checked whether let-7c or let-7a was repressed by androgen in a manner similar to

miR-99a, miR-100 and miR-125b. let-7c, but nor let-7a, was repressed by synthetic androgen

R1881 in a dose dependent manner in LNCaP cells (Fig. 1A), suggesting that the miR-

99a/let7c/125b-2 cluster is responsive to androgen. To test whether androgen represses the transcription of the cluster, we measured the expression of primary miRNAs of the miR-99a/let7c/125b-2 cluster. Pri-miR-99a, pri-let7c and pri-miR-125b-1 were all decreased by androgen (Fig. 1B).

Since the miR-99a/let7c/125b-2 cluster is located in an intron , we examined whether the host gene, LINC00478, is also repressed by androgen. Two transcripts of LINC00478, long (NR_027790) and short (NR_027791), could encode the miR-99a/let7c/125b-2 cluster (Fig. 1C). Six pairs of primers were used to assess androgen regulation of both transcripts (Fig. 1C). F1R1 and F2R2 detect the mature and precursor forms of the long transcript, respectively. F3R3 detects the mature short transcript, while the other three primer pairs detect both transcripts. Both transcripts of LINC00478 were downregulated by androgen, with the long transcript more responsive than the short transcript (Fig. 1C; F1R1 vs. F3R3).

Downregulation of the miR-99a/let7c/125b-2 cluster by androgen requires the presence of AR

We next determine whether AR is required for the androgen-repression of the miR-99a/let7c/125b-2 cluster. As in the AR-positive LNCaP cells, the expression of the miR-99a/let7c/125b-2 cluster was decreased by androgen in AR-positive C4-2 prostate cancer cells (Fig. 2A and Fig. S1A), but not in AR-negative PC-3 prostate cancer cells (Fig. 2B and Fig. S1B). PC-3 cells have a wild type AR gene but do not express AR due to weak transcriptional activity at the AR promoter (27). Ectopic expression of wild type AR in PC-3 cells (Fig. S2B) restored the repression of miR-99a/let7c/125b-2 by androgen (Fig. 2C and Fig. S1C). Conversely, knocking down AR in LNCaP cells by siRNA (Fig. S2A) abolished the repression of miR-99a/let7c/125b-2 by androgen (Fig. 2D and Fig. S1D). These results suggest that AR is required for androgen to repress miR-99a/let7c/125b-2 in prostate cancer cells.

AR binds to the host genes of the miR-99a/let7c/125b-2 cluster

Next we evaluated whether AR directly binds to the host gene of the miR-99a/let7c/125b-2 cluster, LINC00478. AR ChIP-seq data in LNCaP cells in the presence or absence of R1881 have been published (28). Peaks of AR binding were identified using HPeak, a Hidden Markov Model (HMM)-based peak identifying algorithm (http://www.sph.umich.edu/csg/qin/HPeak/). Seven AR binding sites were reported by the ChIP-seq upstream and within the LINC00478 gene: ARBS1-7 (Fig. 3A). ARBS1 and ARBS2 are within 50kb of the transcription start site (TSS) of the long transcript and ARBS3, ARBS4 and ARBS5 are within 50kb of the TSS of the short transcript. Since genome-wide ChIP-seq analyses often have significant false-positive rates, AR ChIP and quantitative real-time PCR was performed to validate whether AR bound to any of these five potential binding sites. Androgen induced AR binding to ARBS1 and ARBS2 (Fig. 3B) to an extent that was similar to the binding to the well-characterized PSA enhancer (AREIII) (10). Significant AR binding or AREs were not seen at the other AR ChIP-seq sites. Interestingly, ARE half-sites were detected at ARBS1 and ARBS2 by ALGGEN-PROMO, a matrix algorithm for predicting transcription factor binding sites based on TRANSFAC (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3) (Fig. S2D). ARBS1 contains "TGTCCT", an exact half site of canonical ARE (5), while ARBS2 contains "GGGACA", which resembles the other half of the canonical ARE ("GGTACA" or "GGAACA") (5).

Polycomb protein EZH2 is involved in AR-repression of the miR-99a/let7c/125b-2 cluster

It has previously been shown that androgen-induced gene expression is mediated by forkhead box protein A1 (FOXA1) and active histone modifications including H3K4 me1 and H3K4me2 (29). However, the mechanisms of androgen-mediated gene repression are more complex. Global ChIP-seq analysis revealed EZH2 binding and a significant enrichment of

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repressive histone mark H3K27me3 around AR-repressed genes, which suggested that EZH2 was involved in AR-repressed gene expression. To test whether EZH2 was responsible for the downregulation of the miR-99a/let7c/125b-2 cluster by androgen treatment, we transfected siRNA against EZH2 in LNCaP cells, and examined the expression of the miR-99a/let7c/125b-2 cluster in the absence or the presence of androgen. Depletion of EZH2 significantly diminished the repression of the miR-99a/let7c/125b-2 cluster by androgen (Fig. 3C).

Target genes of the miR-99a/let7c/125b-2 cluster are enriched in androgen-induced genes

Since the miR-99a/let7c/125b-2 cluster was repressed by androgen, we hypothesized that target genes of miR-99a, let-7c and miR-125b may be upregulated upon androgen treatment as a result of reduced targeting by the miR-99a/let7c/125b-2 cluster. To test this hypothesis, we extracted lists of potential targets of miR-99a, let-7c and miR-125b from TargetScan (version 6.0), a widely used target prediction algorithm of miRNAs. Two lists of androgen-induced genes were obtained from two independent studies (GSE17044 and GSE22606), in which LNCaP cells were treated with R1881or vehicle and gene expression was measured by microarray analysis. To determine which (and how many) androgen-induced genes were also miRNA targets, we intersected predicted targets of each miRNA with androgen-induced genes from each microarray dataset (Table S1, S2 and S3). We performed a Monte Carlo simulation to determine the expected number of miRNA targets in the androgen-induced gene sets at random. The fold enrichment (with p-value) was calculated of the observed number of androgen-induced miRNA targets relative to the random expectation. Potential targets of miR-99a were 4.92 and 6.63 fold enriched in the two respective androgen-induced gene sets, while targets of let-7c and miR-125b were also significantly enriched in androgen-upregulated genes (Table 2).

Table 3 contains the androgen-induced genes that are targets of the miR-99a/let7c/125b-2 cluster that were common between the two microarray datasets used. We selected four target

genes of each miRNA and validated by qRT-PCR that they were induced after androgen treatment. The expression of all of the genes we tested was increased by androgen (Fig. 4A). To test whether the AR-induced genes were also repressed by the miR-99a/let7c/125b-2 cluster, we measured their mRNAs by qRT-PCR after miRNA transfection. At least 75% of the mRNAs tested were significantly decreased by the miR-99a/let7c/125b-2 cluster (Fig. 4B). Since the androgen-repression of the miR-99a/let7c/125b-2 cluster requires AR, we also examined the expression of these mRNAs after knocking-down AR by siRNA. None of the selected genes were stimulated by androgen after AR knock-down (Fig. S3A, B and C). Thus, many genes induced by androgens through AR, are concurrently de-repressed through AR mediated inhibition of the miR-99a/let7c/125b-2 cluster.

IGF1R is a key target of the miR-99a/let7c/125b-2 cluster

Among the androgen-induced target genes of the miR-99a/let7c/125b-2 cluster, factors in the IGF signaling pathway appeared multiple times: insulin-like growth factor 1 receptor (IGF1R) was a target of miR-99a and let-7c, and insulin receptor substrate 2 (IRS2) was a target of let-7c. miR-125b was also predicted to target IGF1R, but at a site that is conserved only among primates. IGF1R is important for the tumorigenesis and progression of prostate cancer, because of its demonstrated roles in angiogenesis, transformation and mitogenesis (30-32). It is frequently overexpressed in prostate tumors and often associated with poor prognosis (33, 34). Several monoclonal antibodies and small molecule inhibitors of IGF1R are currently under investigation in clinical trials for treating metastatic castration-resistant prostate cancer (CRPC) (35, 36). We therefore decided to follow up on IGF1R and first validated that IGF1R was a direct target of the miR-99a/let7c/125b-2 cluster by luciferase reporter assay (Fig. 5A). In the 3'UTR of IGF1R there was one predicted binding site each for miR-99a and miR-125b and three for let-7c (Fig. 5A). It was shown previously that let-7 directly downregulated several genes in

the IGF signaling pathway, including IGF1R, but the report did not clarify whether all three

target sites were active in IGF1R repression (37). Therefore, we tested the effect of each miRNA

target site on IGF1R by dividing the 3'UTR of IGF1R into three fragments and cloning each of

them into a luciferase reporter. The first fragment contained one let-7c binding site, the second

fragment had the miR-125b binding site and another let-7c binding site, and the third fragment

included the miR-99a binding site and the third let-7c binding site (Fig. 5A). In the luciferase

reporter assay, ectopic expression of miR-99a and let-7c but not miR-125b decreased the

luciferase activity, indicating that miR-99a and let-7c directly bound to the 3'UTR of IGF1R and

regulated its expression (Fig. 5A). The two 3'-most binding sites of let-7c responded to the

microRNA, with the most significant regulation coming from the third binding site. The

differential effects of these miRNA binding sites might be due to the secondary structure of the

target mRNA or the binding of other proteins to the target mRNA.

We then checked the expression level of IGF1R after transfecting the miR-

99a/let7c/125b-2 cluster into cells. Overexpression of the miR-99a/let7c/125b-2 cluster

decreased both mRNA and protein level of IGF1R in LNCaP cells (Fig. 5B and C).

Androgen is known to be capable of stimulating the growth of prostate cancer cells, and

members of the miR-99a/let7c/125b-2 cluster have been shown previously to have growth

suppressive functions (19, 38-41). We therefore hypothesized that androgen-repression of the

miR-99a/let7c/125b-2 cluster promoted androgen-dependent growth through the derepression of

IGF1R. To test this hypothesis, we first performed a growth assay in LNCaP cells. Addition of

androgen stimulated proliferation of LNCaP cells (Fig. 5D, CS v.s. 1nM R1881 si-GL2), while

forced expression of miR-99a, let-7c or miR-125b to prevent their downregulation by androgen

blunted the growth stimulatory effect of androgen (Fig. 5D). siRNA against IGF1R generated a

similar phenotype (Fig. 5D), suggesting that miR-99a and let-7c, the two miRNAs we found to

directly regulate IGF1R expression, may inhibit LNCaP cell growth through targeting IGF1R. To test this possibility, we generated a LNCaP cell line that stably expresses IGF1R, which lacks the 3'UTR and is not regulated by miRNAs (Fig. S2C). Overexpression of IGF1R without its 3'UTR rescued the growth defect caused by miR-99a, let-7c or siRNA targeting the 3'UTR of IGF1R, indicating that the growth suppression of LNCaP cells by the miR-99a/let7c/125b-2 cluster in the presence of androgen was primarily due to the downregulation of IGF1R (Fig. 5E). The result also suggests that repression of the miR-99a/let7c/125b-2 cluster by androgen will contribute to prostate cancer growth by relieving IGF1R from microRNA inhibition (Fig. 5F).

Discussion

miRNAs, like other RNA polymerase II driven transcripts, are known to be regulated by various transcription factors . miR-34 and miR-205 both possess tumor suppressive function in cancer cells and are directly induced by the tumor suppressor p53 (42, 43). The expression of miR-17 oncomiR cluster, on the other hand, is known to be driven by oncogenic transcription factor c-myc (44). Similar to p53 and c-myc, androgen receptor has also been shown to regulate miRNA expression (21, 25, 26, 45). Androgen-regulated miRNAs also contribute to castration-resistant prostate cancer (CRPC) (21, 25). Many androgen-induced miRNAs, such as miR-32, miR-148 and miR-21, are oncogenic and upregulated in CRPC compared to benign prostate tissue, while androgen-repressed miRNAs, such as miR-99a and miR-221, tend to be tumor suppressive and are usually downregulated in CRPC (19, 21, 25, 45).

In this paper, we demonstrate that the miR-99a/let7c/125b-2 cluster is repressed by androgen and this can contribute to changes in the gene expression program and cell proliferation induced by androgen. Interestingly, let-7c decreased AR expression by targeting c-myc, a known transcription factor for AR, suggesting that repression of miR-99a/let7c/125b-2 cluster by

androgen may positively feedback and stimulate AR production (46-48). In contrast to our results, S. Inoue's group reported the miR-99a/let7c/125b-2 cluster as an androgen-induced gene in LNCaP cells after 24 hour treatment with R1881 (49). This may be explained by different conditions of androgen treatment and tissue culture, since we harvested cells after 48 hours of androgen-deprivation (charcoal-stripped media and FBS) followed by 48 hours of R1881 treatment. On the other hand, our results are supported by two other independent studies. One study showed that the host gene of the miR-99a/let7c/125b-2 cluster LINC00478 was repressed by androgen in VCaP and VCS2 cells (10). Another study observed androgen-repression of miR-125b by northern blotting in LNCaP cells (25).

According to published ChIP-seq data, there were two AR binding sites (ARBS) within 50kb of the TSS of the long transcript of LINC00478 and three ARBS near the TSS of the short variant. Our AR ChIP-qPCR analysis only confirmed two of these AR binding sites near the promoter of the long transcript (Fig. 3B). However, our RT-qPCR demonstrated that both of the transcripts were repressed by androgen, though the short variant is repressed to a less extent (Fig. 1C; mature transcripts F3R3 vs. F1R1). The modest regulation of the short variant by androgen may be due to its remote location relative to the potential AR enhancers ARBS1 and ARBS2. Other weaker AR enhancers of the short transcript, not captured by the ChIP-seq analysis, may also exist. An interesting twist is that the two functional ARBS have only half-ARE sites. Such half-ARE sites can bind to AR both *in vitro* and *in vivo* (6, 50). Therefore, these two ARE half-sites likely serve as transcriptional enhancers for AR. It is still unknown how AR binds to ARE half-sites. Since AR forms dimer independent of DNA binding, it is possible that only one DNA binding domain (DBD) of the AR dimer binds to the ARE half-site and this binding is stabilized by other DNA binding proteins (51). The AR dimer may also bind to two separate ARE half-sites (ARBS1 and ARBS2) through chromatin looping.

EZH2 (enhancer of zeste 2) is part of the Polycomb Repressive Complex 2 (PRC2) and responsible for the trimethylation of H3K27 on target gene promoters. EZH2 is frequently overexpressed in aggressive tumors including prostate cancer, which is often associated with poor prognosis (52, 53). Knock-down of EZH2 relieved its target genes from repression and inhibited proliferation of prostate cancer cells (54). Global gene repression by AR has been suggested to be mainly mediated by EZH2 and its associated repressive histone mark H3K27me3 (9). Our results are consistent with this suggestion, though androgen still repressed the miR-99a/let7c/125b-2 cluster in cells transfected with si-EZH2. siRNAs cannot completely eliminate the target in the transfected cells and this could account for the residual repression by androgen , but we cannot rule out additional mechanisms that contribute to repression of the miR-99a/let7c/125b-2 cluster by androgen. Many genes repressed by AR and EZH2 promoted cell differentiation and were downregulated in CRPC (9), just as seen with the miR-99a/let7c/125b-2 cluster. The reduced expression of the miR-99a/let7c/125b-2 cluster in CRPC is consistent with upregulation of EZH2 in aggressive prostate cancers.

Insulin-like growth factor 1 receptor (IGF1R) is the primary receptor for IGF-I that also binds to IGF-II and insulin. Ligand-activated IGF1R activates downstream signaling pathways, including the PI3K/Akt pathway, MAPK pathway and STAT3 pathway, resulting in cell proliferation, inhibition of apoptosis and increased motility (30-32). Increased serum level of IGF-I and elevated expression of IGF-I and IGF1R in malignant prostate tumors are often associated with poor prognosis (33, 34). Several monoclonal antibodies and small molecule inhibitors of IGF1R are currently under investigation in clinical trials for treating metastatic castration-resistant prostate cancer (CRPC) (35, 36). IGF-I enhances the nuclear translocation of AR, while androgen promotes the cellular response to IGF-I treatment (30, 55) thereby producing a pro-proliferation positive feedback loop. This increased response to IGF-I treatment in the

presence of androgen is due to increased expression of IGF1R (55). It was suggested that AR

indirectly increased the transcription of IGF1R, possibly through activation of ERK pathway (55,

56), or through androgen-induced expression of KLF6 (Kruppel factor like 6), a transcription

factor for IGF1R (57, 58). Here we report another way by which the expression of IGF1R is

regulated by androgen. Our results suggest that treatment of androgen decreases the expression of

miR-99a and let-7c and in turn derepresses their common target IGF1R at both the mRNA and

protein level. This pathway could also account for conditions where androgen increases the

protein level of IGF1R without an increase in mRNA (58). Consistent with previous studies, we

also confirm the direct regulation of IGF1R by miR-99a and let-7c (37, 59). Furthermore, we

determined that the second and third predicted binding sites in the 3'UTR of IGF1R were the

primary target sites for let-7c.

     While it is generally accepted that androgen-activated AR binds to promoters of many

androgen-induced genes, our results suggest that several of these genes are also targets of

microRNAs that are repressed by androgen-activated AR. For most of these androgen-induced

genes we expect the repression of the targeting microRNA to be a secondary means of increasing

expression of genes whose promoters are directly activated by AR. However, it is noteworthy

that of the 32 targets of the miR-99a/let7c/125b-2 cluster that are induced by androgen and listed

in Table 3, 12 have no AR binding peaks within 50kb of their promoters. Thus it is also possible

that relief of post-transcriptional degradation by miRNAs plays an important role in induction of

at least some genes that are increased by androgen. Although there have been some studies

focusing on miRNAs induced by oncogenes and tumor suppressors, this study highlights the

importance of following microRNAs that may be repressed by such genes. Also gene expression

changes induced by nuclear hormone receptors like AR are usually believed to be due to the

direct action of the receptor on the promoter of the target genes. Our results suggest that some of

the gene induction may be explained by integration of miRNAs repressed by such nuclear receptors into the network.

Taken together, our results identify a regulatory network involving key players in the development of prostate cancer: the transcription factor AR, the tumor suppressive chromatin remodeling factor, EZH2, growths suppressive microRNAs in the miR-99a/let7c/125b-2 cluster, and signal transduction factor IGF1R. Upon androgen treatment, activated AR translocates to the nucleus, where it binds to AREs (ARE half-sites) at the enhancer of a non-coding RNA LINC00478 and recruits the histone methyltransferase EZH2. This results in decreased expression of LINC00478 and the miR-99a/let7c/125b-2 cluster generated from its intron. Downregulation of miR-99a, let-7c and miR-125b leads to derepression of their target genes, including IGF1R, and triggers a series of downstream signaling cascades, which increases proliferation of prostate cancer cells (Fig. 5F). Similarly, in CRPC cells, hyperactivated AR reduces expression of the miR-99a/let7c/125b-2 cluster and increases expression of their targets, which may contribute to prostate cancer progression. This is consistent with the reduced expression of miR-99a, let-7c and miR-125b and increased level of IGF1R seen in prostate cancer cells (19, 21, 33, 34, 39).

Materials and Methods

Cells and Tissues

Human prostate cancer cells LNCaP, C4-2 and PC3 were obtained from ATCC and maintained in RPMI 1640 medium, supplemented with 10% fetal bovine serum. For experiments on androgen responsiveness, cells were cultured in phenol red-free RPMI 1640 medium

supplemented with charcoal:dextran stripped fetal bovine serum (Hyclone) for 48 hours before the addition of the androgen analog R1881 (Perkin-Elmer).

Transfection of siRNA and cell growth assay

Transfection of siRNA or miRNA duplex was performed with Lipofectamine RNAiMax reagent (Invitrogen) as described (60). siRNA was transfected on Day 0 and Day 2 separately, and cells were counted on Day 4 using Countess automated cell counter (Invitrogen).

Chromatin Immunoprecipitation (ChIP) Assay

LNCaP cells were fixed using formaldehyde at RT for 15min and then lysed. Chromatin was sonicated to an average length of 300-500bp. Anti-AR (H-280, santa cruz) and rabbit ChIP grade IgG (Abcam) were used to precipitate DNA fragments. Quantitative real-time PCR was used to analyze AR binding at seven putative AR binding sites. PSA enhancer (ARE3) was used as a positive control (10). The primers used in q-PCR analysis were described in Table S4.

Western blotting

The antibodies used were as follows: anti-IGF1R β (Cell Signaling), anti-AR (Millipore) and anti-β-actin (Sigma). The western blot image was captured by G:Box iChemi XT gel documentation and analysis system. Signal intensity of western blots was quantified with GeneTools from SynGene.

RNA isolation and quantification of miRNA

Total RNA was extracted using TRIzol (Invitrogen). 1μg total RNA was reverse transcribed using NCode miRNA First-Strand cDNA Synthesis kit (Invitrogen). The expression level of miRNAs was measured by quantitative PCR using NCode SYBR GreenER miRNA qPCR kit in triplicate. U6 small nuclear RNA (snU6) was used to normalize the expression data of miRNAs (19).

Luciferase reporter assay

The 3'-UTR fragments of IGF1R containing miR-99, let-7c and miR-125b binding sites were cloned into a modified vector pRL-CMV (19). The mutations were made to the miRNA binding sites in the 3'UTR-MUT clones. The primers used in 3'UTR or 3'UTR-MUT cloning are described in Table S4. The luciferase reporter assay was performed as previously described (60).

HPeak analysis of ChIP-seq data

We analyzed the AR ChIP-seq data in LNCaP cells in the presence or the absence of androgen. AR ChIP-seq data were downloaded from GEO database (GSM353644 and GSM353643). HPeak version 2.1 (61) was used to find genomic regions enriched for androgen-induced AR binding in LNCaP cells.

Enrichment analysis of miRNA targets in androgen-induced gene sets

The lists of predicted targets of miR-99a, let-7c and miR-125b were downloaded from TargetScan (http://www.targetscan.org/, version 6.0). We only considered the targets where the sites were broadly conserved among vertebrates. Two sets of androgen-induced genes in LNCaP cells were generated from two independent microarray analyses (GSE22606 and GSE17044). All genes with ≥1.2 fold induction by androgen were included. To determine if putative targets of miR-99a/let7c/125b-2 cluster were enriched in androgen-induced gene sets, we first generated the "observed number" of androgen-induced genes that are also miRNA targets by intersecting the potential targets of miR-99a/let7c/125b-2 with each androgen-induced gene set. The randomly "expected number" of androgen-induced genes that are also miRNA targets given the number of miRNA targets were determined using 1000 rounds of Monte Carlo simulation, assuming that miRNA targets were uniformly distributed in the total database of genes. The significance of any enrichment of "observed" to "expected" is also given by the Monte Carlo simulation as the proportion of simulation rounds that were more extreme than the observation.

Conflict of interests

The authors declare no conflict of interest.

Acknowledgements

References

1.   Richter E, Srivastava S, Dobi A. Androgen receptor and prostate cancer. Prostate Cancer Prostatic Dis 2007;10(2):114-8.
2.   Denayer S, Helsen C, Thorrez L, Haelens A, Claessens F. The rules of DNA recognition by the androgen receptor. Mol Endocrinol 2010;24(5):898-913.
3.   Nelson PS, Clegg N, Arnold H, Ferguson C, Bonham M, White J, et al. The program of androgen-responsive genes in neoplastic prostate epithelium. Proc Natl Acad Sci U S A 2002;99(18):11890-5.
4.   Wang Q, Li W, Liu XS, Carroll JS, Janne OA, Keeton EK, et al. A hierarchical network of transcription factors governs androgen receptor-dependent prostate cancer growth. Mol Cell 2007;27(3):380-92.
5.   Takayama K, Kaneshiro K, Tsutsumi S, Horie-Inoue K, Ikeda K, Urano T, et al. Identification of novel androgen response genes in prostate cancer cells by coupling chromatin immunoprecipitation and genomic microarray analysis. Oncogene 2007;26(30):4453-63.
6.   Massie CE, Adryan B, Barbosa-Morais NL, Lynch AG, Tran MG, Neal DE, et al. New androgen receptor genomic targets show an interaction with the ETS1 transcription factor. EMBO Rep 2007;8(9):871-8.
7.   Gonit M, Zhang J, Salazar M, Cui H, Shatnawi A, Trumbly R, et al. Hormone depletion-insensitivity of prostate cancer cells is supported by the AR without binding to classical response elements. Mol Endocrinol 2011;25(4):621-34.
8.   Ngan S, Stronach EA, Photiou A, Waxman J, Ali S, Buluwela L. Microarray coupled to quantitative RT-PCR analysis of androgen-regulated genes in human LNCaP prostate cancer cells. Oncogene 2009;28(19):2051-63.
9.   Zhao JC, Yu J, Runkle C, Wu L, Hu M, Wu D, et al. Cooperation between Polycomb and androgen receptor during oncogenic transformation. Genome Res 2012;22(2):322-31.
10.   Cai C, He HH, Chen S, Coleman I, Wang H, Fang Z, et al. Androgen receptor gene expression in prostate cancer is directly suppressed by the androgen receptor through recruitment of lysine-specific demethylase 1. Cancer Cell 2011;20(4):457-71.
11.   Liu YN, Liu Y, Lee HJ, Hsu YH, Chen JH. Activated androgen receptor downregulates E-cadherin gene expression and promotes tumor metastasis. Mol Cell Biol 2008;28(23):7096-108.
12.   Jiang J, Pan Y, Regan KM, Wu C, Zhang X, Tindall DJ, et al. Androgens repress expression of the F-box protein Skp2 via p107 dependent and independent mechanisms in LNCaP prostate cancer cells. Prostate 2012;72(2):225-32.
13.   Lanzino M, Sisci D, Morelli C, Garofalo C, Catalano S, Casaburi I, et al. Inhibition of cyclin D1 expression by androgen receptor in breast cancer cells--identification of a novel androgen response element. Nucleic Acids Res 2010;38(16):5351-65.
14.   Baniwal SK, Khalid O, Sir D, Buchanan G, Coetzee GA, Frenkel B. Repression of Runx2 by androgen receptor (AR) in osteoblasts and prostate cancer cells: AR binds Runx2 and abrogates its recruitment to DNA. Mol Endocrinol 2009;23(8):1203-14.
15.   Curtin D, Jenkins S, Farmer N, Anderson AC, Haisenleder DJ, Rissman E, et al. Androgen suppression of GnRH-stimulated rat LHbeta gene transcription occurs through Sp1 sites in the distal GnRH-responsive promoter region. Mol Endocrinol 2001;15(11):1906-17.

16.   Nelius T, Filleur S, Yemelyanov A, Budunova I, Shroff E, Mirochnik Y, et al. Androgen receptor targets NFkappaB and TSP1 to suppress prostate tumor growth in vivo. Int J Cancer 2007;121(5):999-1008.

17.   Lu J, Getz G, Miska EA, Alvarez-Saavedra E, Lamb J, Peck D, et al. MicroRNA expression profiles classify human cancers. Nature 2005;435(7043):834-8.

18.   Mitchell PS, Parkin RK, Kroh EM, Fritz BR, Wyman SK, Pogosova-Agadjanyan EL, et al. Circulating microRNAs as stable blood-based markers for cancer detection. Proc Natl Acad Sci U S A 2008;105(30):10513-8.

19.   Sun D, Lee YS, Malhotra A, Kim HK, Matecic M, Evans C, et al. miR-99 family of MicroRNAs suppresses the expression of prostate-specific antigen and prostate cancer cell proliferation. Cancer Res 2011;71(4):1313-24.

20.   Tong AW, Fulgham P, Jay C, Chen P, Khalil I, Liu S, et al. MicroRNA profile analysis of human prostate cancers. Cancer Gene Ther 2009;16(3):206-16.

21.   Jalava SE, Urbanucci A, Latonen L, Waltering KK, Sahu B, Janne OA, et al. Androgen-regulated miR-32 targets BTG2 and is overexpressed in castration-resistant prostate cancer. Oncogene 2012.

22.   Waltering KK, Porkka KP, Jalava SE, Urbanucci A, Kohonen PJ, Latonen LM, et al. Androgen regulation of micro-RNAs in prostate cancer. Prostate 2011;71(6):604-14.

23.   Wang WL, Chatterjee N, Chittur SV, Welsh J, Tenniswood MP. Effects of 1alpha,25 dihydroxyvitamin D3 and testosterone on miRNA and mRNA expression in LNCaP cells. Mol Cancer 2011;10:58.

24.   Gommans WM, Berezikov E. Controlling miRNA regulation in disease. Methods Mol Biol 2012;822:1-18.

25.   Ribas J, Ni X, Haffner M, Wentzel EA, Salmasi AH, Chowdhury WH, et al. miR-21: an androgen receptor-regulated microRNA that promotes hormone-dependent and hormone-independent prostate cancer growth. Cancer Res 2009;69(18):7165-9.

26.   Fletcher CE, Dart DA, Sita-Lumsden A, Cheng H, Rennie PS, Bevan CL. Androgen-regulated processing of the oncomir MiR-27a, which targets Prohibitin in prostate cancer. Hum Mol Genet 2012;21(14):3112-27.

27.   Chlenski A, Nakashiro K, Ketels KV, Korovaitseva GI, Oyasu R. Androgen receptor expression in androgen-independent prostate cancer cell lines. Prostate 2001;47(1):66-75.

28.   Yu J, Mani RS, Cao Q, Brenner CJ, Cao X, Wang X, et al. An integrated network of androgen receptor, polycomb, and TMPRSS2-ERG gene fusions in prostate cancer progression. Cancer Cell 2010;17(5):443-54.

29.   Lupien M, Eeckhoute J, Meyer CA, Wang Q, Zhang Y, Li W, et al. FoxA1 translates epigenetic signatures into enhancer-driven lineage-specific transcription. Cell 2008;132(6):958-70.

30.   Kojima S, Inahara M, Suzuki H, Ichikawa T, Furuya Y. Implications of insulin-like growth factor-I for prostate cancer therapies. Int J Urol 2009;16(2):161-7.

31.   Papatsoris AG, Karamouzis MV, Papavassiliou AG. Novel insights into the implication of the IGF-1 network in prostate cancer. Trends Mol Med 2005;11(2):52-5.

32.   Ozkan EE. Plasma and tissue insulin-like growth factor-I receptor (IGF-IR) as a prognostic marker for prostate cancer and anti-IGF-IR agents as novel therapeutic strategy for refractory cases: a review. Mol Cell Endocrinol 2011;344(1-2):1-24.

33.   Cardillo MR, Monti S, Di Silverio F, Gentile V, Sciarra F, Toscano V. Insulin-like growth factor (IGF)-I, IGF-II and IGF type I receptor (IGFR-I) expression in prostatic cancer. Anticancer Res 2003;23(5A):3825-35.

34.   Liao Y, Abel U, Grobholz R, Hermani A, Trojan L, Angel P, et al. Up-regulation of insulin-like growth factor axis components in human primary prostate cancer correlates with tumor grade. Hum Pathol 2005;36(11):1186-96.

35.   Gennigens C, Menetrier-Caux C, Droz JP. Insulin-Like Growth Factor (IGF) family and prostate cancer. Crit Rev Oncol Hematol 2006;58(2):124-45.

36.   Antonarakis ES, Carducci MA, Eisenberger MA. Novel targeted therapeutics for metastatic castration-resistant prostate cancer. Cancer Lett 2010;291(1):1-13.

37.   Zhu H, Shyh-Chang N, Segre AV, Shinoda G, Shah SP, Einhorn WS, et al. The Lin28/let-7 axis regulates glucose metabolism. Cell 2011;147(1):81-94.

38.   Guan Y, Yao H, Zheng Z, Qiu G, Sun K. MiR-125b targets BCL3 and suppresses ovarian cancer proliferation. Int J Cancer 2011;128(10):2274-83.

39.   Nadiminty N, Tummala R, Lou W, Zhu Y, Shi XB, Zou JX, et al. MicroRNA let-7c is downregulated in prostate cancer and suppresses prostate cancer growth. PLoS One 2012;7(3):e32832.

40.   Huang L, Luo J, Cai Q, Pan Q, Zeng H, Guo Z, et al. MicroRNA-125b suppresses the development of bladder cancer by targeting E2F3. Int J Cancer 2011;128(8):1758-69.

41.   Mueller AC, Sun D, Dutta A. The miR-99 family regulates the DNA damage response through its target SNF2H. Oncogene 2012.

42.   He L, He X, Lim LP, de Stanchina E, Xuan Z, Liang Y, et al. A microRNA component of the p53 tumour suppressor network. Nature 2007;447(7148):1130-4.

43.   Piovan C, Palmieri D, Di Leva G, Braccioli L, Casalini P, Nuovo G, et al. Oncosuppressive role of p53-induced miR-205 in triple negative breast cancer. Mol Oncol 2012;6(4):458-72.

44.   O'Donnell KA, Wentzel EA, Zeller KI, Dang CV, Mendell JT. c-Myc-regulated microRNAs modulate E2F1 expression. Nature 2005;435(7043):839-43.

45.   Zheng C, Yinghao S, Li J. MiR-221 expression affects invasion potential of human prostate carcinoma cell lines by targeting DVL2. Med Oncol 2012;29(2):815-22.

46.   Grad JM, Dai JL, Wu S, Burnstein KL. Multiple androgen response elements and a Myc consensus site in the androgen receptor (AR) coding region are involved in androgen-mediated up-regulation of AR messenger RNA. Mol Endocrinol 1999;13(11):1896-911.

47.   Lee JG, Zheng R, McCafferty-Cepero JM, Burnstein KL, Nanus DM, Shen R. Endothelin-1 enhances the expression of the androgen receptor via activation of the c-myc pathway in prostate cancer cells. Mol Carcinog 2009;48(2):141-9.

48.   Nadiminty N, Tummala R, Lou W, Zhu Y, Zhang J, Chen X, et al. MicroRNA let-7c suppresses androgen receptor expression and activity via regulation of Myc expression in prostate cancer cells. J Biol Chem 2012;287(2):1527-37.

49.   Takayama K, Tsutsumi S, Katayama S, Okayama T, Horie-Inoue K, Ikeda K, et al. Integration of cap analysis of gene expression and chromatin immunoprecipitation analysis on array reveals genome-wide androgen receptor signaling in prostate cancer cells. Oncogene 2011;30(5):619-30.

50.   Bolton EC, So AY, Chaivorapol C, Haqq CM, Li H, Yamamoto KR. Cell- and gene-specific regulation of primary target genes by the androgen receptor. Genes Dev 2007;21(16):2005-17.

51.   van Royen ME, van Cappellen WA, de Vos C, Houtsmuller AB, Trapman J. Stepwise androgen receptor dimerization. J Cell Sci 2012;125(Pt 8):1970-9.

52.   Yu J, Rhodes DR, Tomlins SA, Cao X, Chen G, Mehra R, et al. A polycomb repression signature in metastatic prostate cancer predicts cancer outcome. Cancer Res 2007;67(22):10657-63.

53.   Kleer CG, Cao Q, Varambally S, Shen R, Ota I, Tomlins SA, et al. EZH2 is a marker of aggressive breast cancer and promotes neoplastic transformation of breast epithelial cells. Proc Natl Acad Sci U S A 2003;100(20):11606-11.

54.   Varambally S, Dhanasekaran SM, Zhou M, Barrette TR, Kumar-Sinha C, Sanda MG, et al. The polycomb group protein EZH2 is involved in progression of prostate cancer. Nature 2002;419(6907):624-9.

55.   Pandini G, Mineo R, Frasca F, Roberts CT, Jr., Marcelli M, Vigneri R, et al. Androgens up-regulate the insulin-like growth factor-I receptor in prostate cancer cells. Cancer Res 2005;65(5):1849-57.

56.   Pandini G, Genua M, Frasca F, Vigneri R, Belfiore A. Sex steroids upregulate the IGF-1R in prostate cancer cells through a nongenotropic pathway. Ann N Y Acad Sci 2009;1155:263-7.

57.   Rubinstein M, Idelman G, Plymate SR, Narla G, Friedman SL, Werner H. Transcriptional activation of the insulin-like growth factor I receptor gene by the Kruppel-like factor 6 (KLF6) tumor suppressor protein: potential interactions between KLF6 and p53. Endocrinology 2004;145(8):3769-77.

58.   Wu JD, Haugk K, Woodke L, Nelson P, Coleman I, Plymate SR. Interaction of IGF signaling and the androgen receptor in prostate cancer progression. J Cell Biochem 2006;99(2):392-401.

59.   Chen Z, Jin Y, Yu D, Wang A, Mahjabeen I, Wang C, et al. Down-regulation of the microRNA-99 family members in head and neck squamous cell carcinoma. Oral Oncol 2012;48(8):686-91.

60.   Kim HK, Lee YS, Sivaprasad U, Malhotra A, Dutta A. Muscle-specific microRNA miR-206 promotes muscle differentiation. J Cell Biol 2006;174(5):677-87.

61.   Qin ZS, Yu J, Shen J, Maher CA, Hu M, Kalyana-Sundaram S, et al. HPeak: an HMM-based algorithm for defining read-enriched regions in ChIP-Seq data. BMC Bioinformatics 2010;11:369.

Figure Legends

Figure 1. miR-99a/let7c/125b-2 cluster and its host gene LINC00478 are repressed by androgen.

A. qPCR was used to measure the expression level of miRNAs. The values were normalized to that of U6sn. Mean±s.d. n=3. The expression level under no androgen treatment is set as 1. LNCaP cells were treated with charcoal-stripped serum for 48 hrs followed by treatment with indicated concentrations of R1881. **B.** The expression of primary miRNAs were measured by qPCR and normalized to β-actin. Rest as in Fig. 1A. **C.** Six pairs of primers were used to measure the expression of the two variant transcripts of LINC00478 at different concentrations of androgen. Schematic at the top shows the two transcripts of LINC00478 with the exons indicated by black boxes and the microRNAs shown by vertical lines. Primer pairs are indicated by horizontal lines. F1R1 and F3R3 amplify across splice junctions of the mature RNA. qPCR value was normalized to GAPDH. Rest as in Fig. 1A

Figure 2. AR is required for androgen-repression of miR-99a/let7c/125b-2 cluster.

A. The expression of primary miRNAs in C4-2 cells were measured by qPCR and normalized to β-actin. Rest as in Fig. 1A. **B.** The expression of primary miRNAs in PC-3 cells. Rest as in Fig. 2A. **C.** The expression of primary miRNAs in PC-3 cells expressing wild type AR. Rest as in Fig. 2A. **D.** The expression of primary miRNAs in LNCaP cells after knocking down AR by siRNA. LNCaP cells were treated with no androgen, 100pM or 1nM R1881. Rest as in Fig. 1A.

Figure 3. AR directly binds to host gene of miR-99a/let7c/125b-2 cluster and the repression is attenuated when EZH2 is knocked down.

A. The AR binding sites at the LINC00478 locus from published AR ChIP-seq data are shown. Seven sites shown as vertical lines are labeled as BS1-7. The two primary transcripts and the microRNAs are shown. **B.** AR ChIP was performed and followed by qPCR. Primers were

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designed to detect AR ChIP-seq peaks ARBS1-5. A PSA enhancer containing an ARE was used

as a positive control. ChIP grade IgG antibody was used as negative control. The mean and

standard deviation from three independent experiments are indicated. The value was expressed as

percentage of input DNA. The dashed line (---) marks the background threshold at 0.05% of

input DNA. The statistical difference between "–"and "+" R1881 for AR ChIP was assessed by t-

test. * indicated p-value less than 10^{-6}. **C.** The expression of primary miRNAs in LNCaP cells

were measured by qPCR and normalized to GAPDH. LNCaP cells were transfected with siRNA

against EZH2 or control si-GL2, and treated without or with 1nM R1881. Mean±s.d. n=3. The

expression in no androgen is set as 1. * indicates p-value of difference from si-GL2 <0.05 and

** indicates p-value <0.01.

Figure 4. Validation of androgen-induced targets of miR-99a/let7c/125b-2 cluster.

A. The expression of four predicted targets each of miR-99a, let-7c or miR-125b respectively

was measured by RT-qPCR and normalized to GAPDH. LNCaP cells grown in charcoal stripped

serum were treated with 0pM, 100pM or 1nM R1881. Mean±s.d. n=3. The expression in no

androgen is set as 1. **B.** The expression of targets of miR-99a/let7c/125b-2 cluster after

transfecting LNCaP cells with miR-99a, let-7c, miR-125b miRNA duplex or control siRNA

duplex si-GL2. Expression was measured by RT-qPCR and normalized to GAPDH. Rest as in

Fig. 4A. * indicates p-value of difference from si-GL2 <0.01.

Figure 5. IGF1R is a key target molecule regulated by miR-99a/let7c/125b-2 cluster.

A. Top: The 3'UTR of IGF1R, the relative positions of each miRNA binding site and the

fragments inserted in the luciferase vectors . * indicates miRNA binding sites validated by

luciferase assays. Bottom: Luciferase assay was performed with control luciferase vector, vector

with indicated 3'UTR fragment of IGF1R, or 3'UTR fragments with mutation in the predicted

target sites (indicated by MUT). The ratio of the renilla luciferase to firefly luciferase

(transfection control) was normalized to that in the si-GL2 transfection. Mean±s.d. n=3 * indicates p-value of difference from si-GL2 <0.005 and ** indicates p-value <0.001. **B.** The level of IGF1R mRNA after transfecting miRNA duplex mixture of miR-99a and let-7c (marked as miRs) or control si-GL2 was measured by RT-qPCR and normalized to β-actin. Mean±s.d. n=3. The value in si-GL2 treated cells was set as 1. **C.** Western blot was used to detect IGF1R protein after transfecting miR-99a, let-7c, miRNA duplex mixture of miR-99a and let-7c (miRs) or control si-GL2. β-actin was used as a loading control. **D.** LNCaP cells were treated with no androgen (NA) or 1nM R1881 during the experiment. miR-99a, let-7c, miR-125b, siRNA against 3'UTR of IGF1R or si-GL2 was transfected twice at 0 hr and 48 hrs. At 96 hrs, cell number was counted using an automated cell counter. The mean and standard deviation from triplicate samples are shown. **E.** miR-99a, let-7c, siRNA against 3'UTR of IGF1R or si-GL2 was transfected twice at 0 hr and 48 hrs. LNCaP cells were treated with 1nM R1881 during the experiment. At 96 hrs, cell number was counted in an automated cell counter. The mean and standard deviation from triplicate samples are shown. **F.** Schematic of how the repression of miR-99a/let-7c/miR-125b-2 by androgen stimulates prostate epithelial cell proliferation.

Table 1. Two miRNA clusters containing miR-99a, miR-100 and miR-125b.

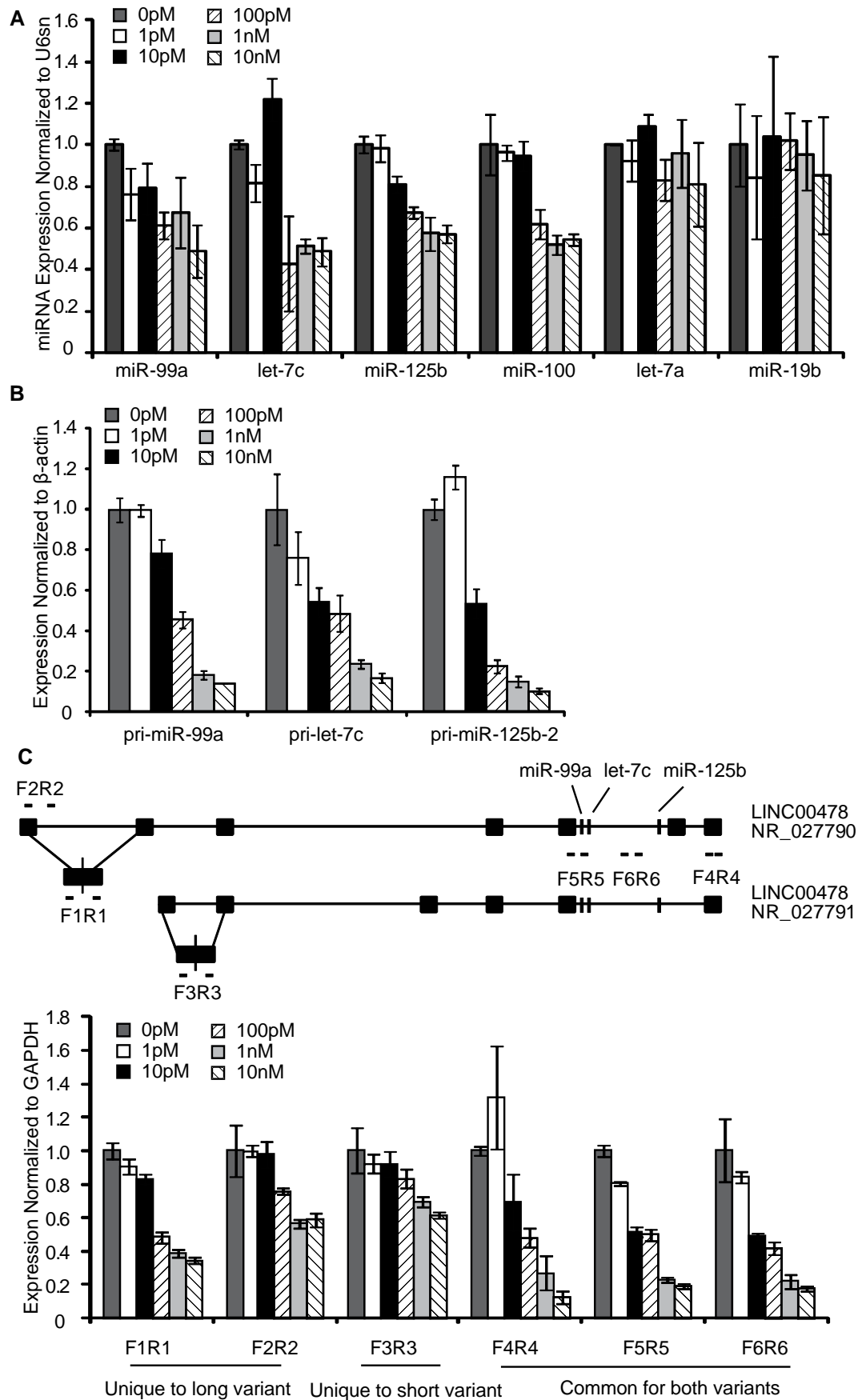| Cluster Name	miRNAs	Genomic Locus	Host Gene
mir-99a/let-7c/miR-125b-2	miR-99a	chr21	LINC00478
	let-7c		
	miR-125b-2		
mir-100/let-7a-2/miR-125b-1	miR-100	chr11	MIR100HG
	let-7a-2		
	miR-125b-1		

Table 2. Enrichment of target genes of miR-99alet7c125b-2 cluster in androgen-induced gene sets. (by Monte Carlo Simulation)

Dataset 1	Number of miRNA targets	Observed Number of Androgen-induced miRNA targets	Expected Number of Androgen-induced genes	Fold Enrichment (Observed/Expected)	P-value
let-7c	845	58	37.0±6.12	1.58	0.001
miR-125b	626	43	27.5±5.24	1.64	0.003
miR-99a	38	8	1.7±1.22	4.92	0.001
Dataset 2					
let-7c	845	28	14.0±3.79	1.89	0.003
miR-125b	626	14	10.3±3.07	1.3	0.149
miR-99a	38	4	0.6±0.75	6.63	0.004

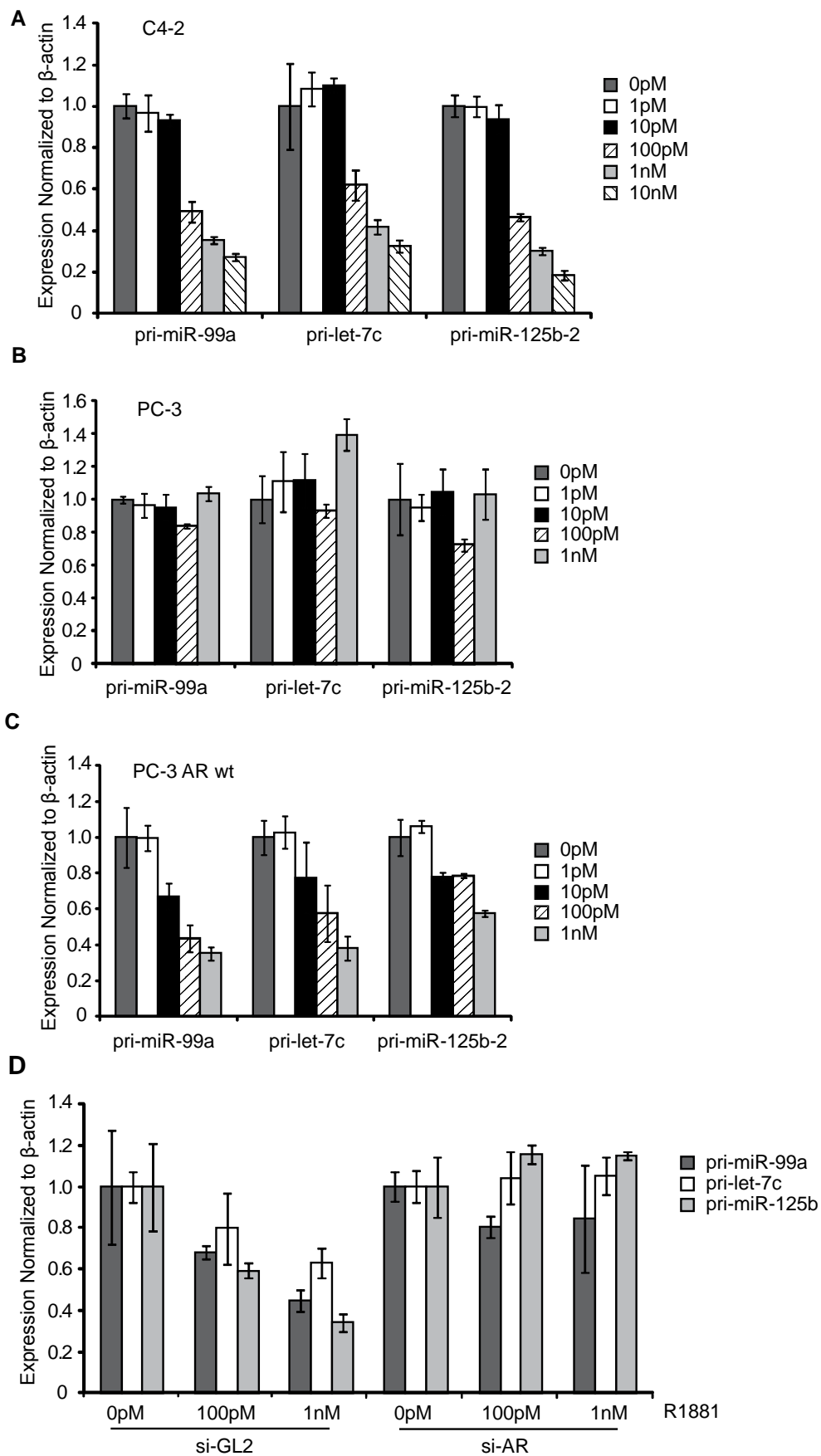**Table 3. Androgen-induced targets genes of miR-99alet7c125b-2 Cluster**

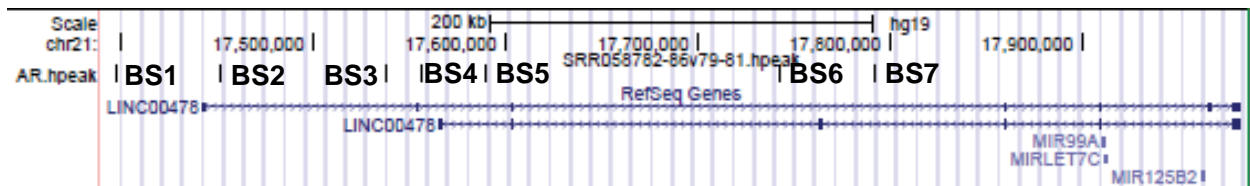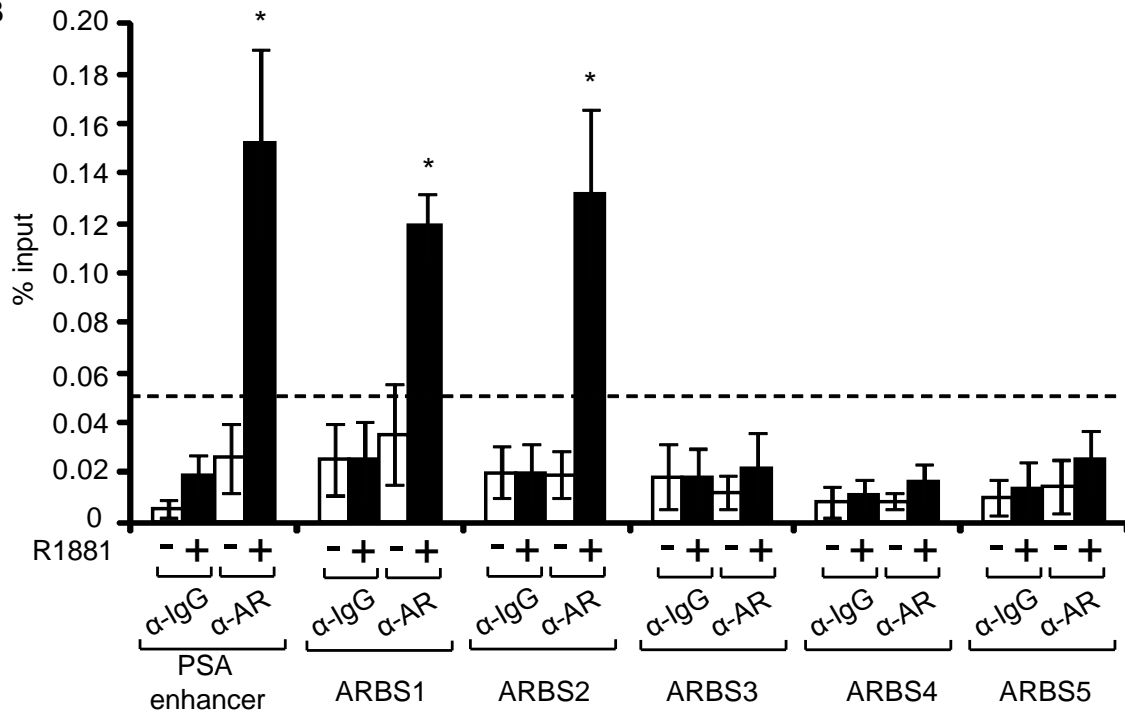| Gene Name	Gene Description	Fold change (GSE22606/GSE17044)
miR-99a targets		
FZD8	frizzled homolog 8 (Drosophila)	1.40/1.27
FZD5	frizzled homolog 5 (Drosophila)	2.77/1.25
FKBP5	FK506 binding protein 5	1.92/1.47
IGF1R	insulin-like growth factor 1 receptor	1.60/1.46
let-7c targets		
LRIG1	leucine-rich repeats and immunoglobulin-like domains 1	1.25/1.30
GREB1	GREB1 protein	1.37/1.68
BCAP29	B-cell receptor-associated protein 29	1.83/1.46
ADRB1	adrenergic, beta-1-, receptor	1.67/1.42
SLC1A4	solute carrier family 1 (glutamate/neutral amino acid transporter), member 4	1.89/1.36
IRS2	insulin receptor substrate 2	1.39/1.25
IGF1R	insulin-like growth factor 1 receptor	1.60/1.46
C14orf28	chromosome 14 open reading frame 28	1.28/1.23
TMPRSS2	transmembrane protease, serine 2	1.60/1.52
EDEM1	ER degradation enhancer, mannosidase alpha-like 1	1.23/1.24
EDEM3	ER degradation enhancer, mannosidase alpha-like 3	1.47/1.34
PNKD	paroxysmal nonkinesigenic dyskinesia	1.26/1.23
DNAJB9	DnaJ (Hsp40) homolog, subfamily B, member 9	1.46/1.32
GALE	UDP-galactose-4-epimerase	1.24/1.25
CPEB3	cytoplasmic polyadenylation element binding protein 3	1.46/1.27
ERO1L	ERO1-like (S. cerevisiae)	1.60/1.24
ANKH	ankylosis, progressive homolog (mouse)	1.91/1.37
STYK1	serine/threonine/tyrosine kinase 1	1.21/1.21
miR-125b targets		
LIFR	leukemia inhibitory factor receptor alpha	2.07/1.41
ANKH	ankylosis, progressive homolog (mouse)	1.91/1.37
MAF	v-maf musculoaponeurotic fibrosarcoma oncogene homolog (avian)	2.28/1.52
ABCC4	ATP-binding cassette, sub-family C (CFTR/MRP), member 4	2.13/1.29
GTPBP2	GTP binding protein 2	1.22/1.32
ALDH1A3	aldehyde dehydrogenase 1 family, member A3	1.35/1.28
DNAJC10	DnaJ (Hsp40) homolog, subfamily C, member 10	1.61/1.30
FLVCR2	feline leukemia virus subgroup C cellular receptor family, member 2	1.41/1.24
EDEM1	ER degradation enhancer, mannosidase alpha-like 1	1.23/1.24
SLC1A5	solute carrier family 1 (neutral amino acid transporter), member 5	1.21/1.22

Sun et al_Figure 2

A

B

C

Sun et al_Figure 5